

**miR-451a Modulates Endometriotic Lesion and Cellular Proliferation
through c-Myc-RPLP1 Regulation:
A Novel Pathway in Endometriosis Pathogenesis**

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Abstract

Endometriosis is a chronic, debilitating inflammatory disease. It is characterized by ectopic formation of estrogen-sensitive endometrial glands and stroma mostly on the surface of the ovaries, fallopian tubes, and pelvic cavity. Major complaints of endometriosis patients are pelvic pain, dysmenorrhea and infertility, which decrease the quality of life of these women. Non-steroidal anti-inflammatory medications and oral contraceptives are the first line of treatment to alleviate the symptoms and regress the lesions. The diagnosis of the disease is a hurdle; there are no specific markers for endometriosis, and the only approach to confirm the disease is by laparoscopy. The reason for limited treatment and diagnosis options is the lack of complete understanding of endometriosis pathophysiology. Retrograde menstruation is one of the most accepted theories, however, considering that retrograde menstruation is a normal process in most women of reproductive age, the causes of the establishment and survival of endometriotic tissue in these patients is not clear. Several studies suggested that microRNAs may play a significant role in endometriosis development. MicroRNAs are small non-coding RNAs that stimulate or inhibit the transcription of specific gene targets. Mis-expression of microRNAs has been established in endometriosis and is linked to its cellular phenotypes. miRNA-451 (now referred to as miR-451a), is one of the most differential expressed miRNAs in endometriotic lesions from women with endometriosis. It is well-established that miR-451a modulates cell survival, but its potential role in endometriosis is poorly understood. We have previously demonstrated that miR-451a regulates expression of factors essential for endometriotic lesion survival. The objective of this project was to further explore the mechanisms and mediators through which miR-451a may modulate endometriotic lesion and cell survival. To accomplish our goal, we over-expressed miR-451a in the immortalized endometriotic epithelial 12Z cell line and screened differentially expressed

proteins by 2-DiGE analysis. As a result, RPLP1, Ribosomal Protein Large P1, was found to be decreased in 12Z cells which over-expressed miR-451a. RPLP1 is a component of 60S ribosomal subunit which aids in the translation elongation process and is speculated to drive cell proliferation. Thus, *the overall hypothesis of this research project is that RPLP1 is a target of miR-451a and its expression is related to the proliferation and survival of endometriotic ectopic lesions.* To test this, we utilized matched human eutopic and ectopic endometrium (endometriotic lesions). We found that in endometriotic lesion samples, the majority 52/77 samples) expressed elevated levels of RPLP1 mRNA compared to matched eutopic tissues. RPLP1 protein was also increased in ectopic lesion tissue compared to matched eutopic endometrium. In addition, a positive correlation was observed between RPLP1 mRNA expression and that of the proliferative marker, cyclin E1 (CCNE1). To examine the function of RPLP1 in 12Z cells, we knocked down RPLP1 expression using siRNA technology and observed a significant decrease in the expression of CCNE1, and a concurrent increase in PTEN (a pro-apoptotic marker). In addition, RPLP1 knockdown by shRNA demonstrated an approximate 90% reduction in cell survival of 12Z cells compared to those infected with non-targeting shRNA. To explore the mechanism of miR-451a in modulating RPLP1 expression, we conducted a literature review and bio-informatic prediction tools search. This revealed that c-Myc was a putative direct target of miR-451a which may potentially regulate RPLP1 expression. C-Myc is a transcriptional factor that regulates the expression of many genes involved in cell proliferation and tumorigenesis. To investigate the possible regulation of c-Myc by miR-451a, we first examined the expression of c-Myc in miR-451a transfected 12Z cells and found significant downregulation on the protein and mRNA level. Furthermore, knockdown of c-Myc by siRNA transfection resulted in a significant reduction of RPLP1 transcript and protein in 12Z cells. To examine the relationship between c-Myc and RPLP1 in endometriotic lesion tissue,

we first examined the expression of c-Myc transcript in human eutopic and ectopic endometrial samples. We found that the mean fold change of c-Myc expression was four-fold higher in ectopic lesions compared to eutopic tissues. Expression of c-Myc and RPLP1 mRNA were positively correlated. Collectively, these data confirm our hypothesis that RPLP1 is a player in mediating endometriotic cell/lesion survival. In addition, our data revealed a novel pathway which involve that miR-451a directly target c-Myc, which in turn may control the expression of RPLP1. Outcomes from this research may lead to more specific approaches to treating endometriosis which may avoid use of anti-estrogen therapies and their unwanted side effects

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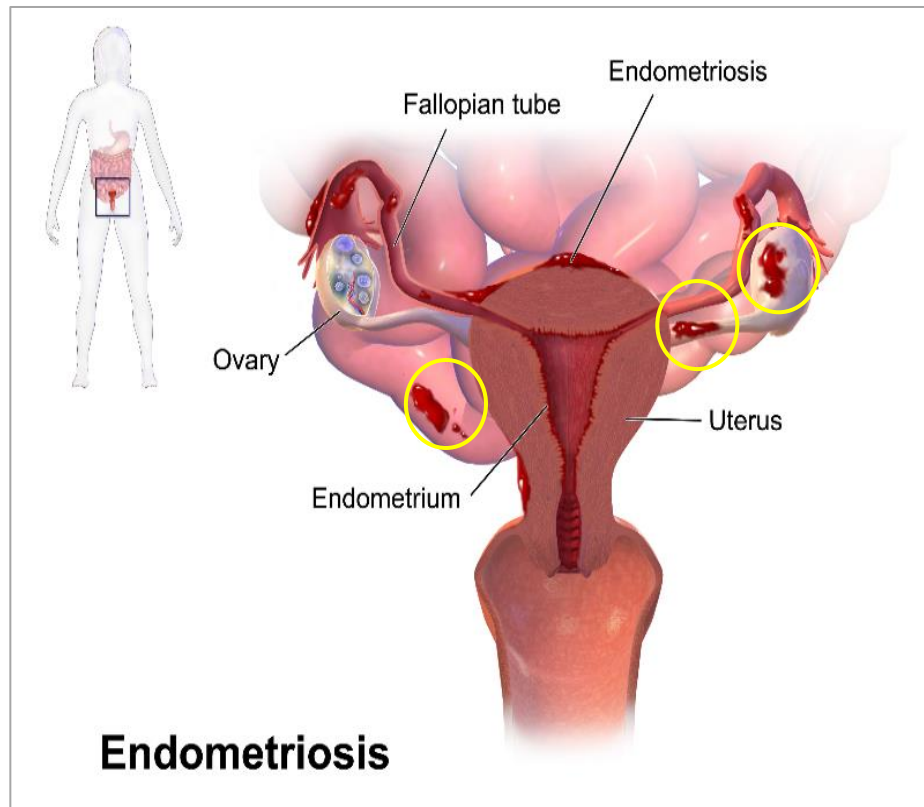
Chapter I: Endometriosis: A General Introduction

I. Definition:

The endometrium is composed of a functionalis layer, which consists of glandular epithelium and stromal cells, that sheds during menstruation, and a basalis layer that contains the stem cells which are the source of endometrial tissue and stay intact during menstruation [1]. Endometriosis is thought to develop due to “reverse menstruation” of endometrial tissue (glands and stroma) through the fallopian tubes, “seeding” of the peritoneal cavity with this retrogradely shed tissue and eventual attachment and survival of the tissue. Lesions are most commonly found on the surfaces of the peritoneal cavity, ovaries, and rectovaginal fascia (Figure 1). Other less common locations include pericardium, intestines, and diaphragm. (Figure 1) [2, 3] . It is estimated that around 10% of women of reproductive age are affected by endometriosis. The condition is mostly associated with pelvic pain and impaired fertility [4]. In addition, endometriosis may cause urinary or bowel discomfort to patients due to the buildup of the endometriotic lesion tissues on these organs [5, 6]. In the United States, endometriosis is considered a major cause of hysterectomy and hospitalization. As a consequence, endometriosis affects the quality of life of the affected individual, as well as places a financial burden of total annual medical care costs which total around \$ 22 billion in the US alone [7].

Figure 1: Endometriotic lesions locations. Endometriosis is characterized by the development of ectopic endometrial tissues in different locations outside of the uterine cavity (marked by yellow ovals). (Figure was adapted from Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". WikiJournal of Medicine 1 (2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436.)[3]

Figure 1



II. Characteristics, Classifications and Clinical Presentation:

1. Epidemiology:

Endometriosis is a chronic disease that affects 5-10% of women of reproductive age [8]. However, it is believed that this percentage may be under-estimated. The manifestations associated with endometriosis can be undermined by patients and physicians, or confused with other diseases such as irritable bowel syndrome (IBS), fibromyalgia, and other chronic pelvic pain syndromes [9]. In addition, the only method to confirm presence of the disease is laparoscopy or laparotomy and histological confirmation of endometriotic lesions (i.e., endometrial glandular and stromal tissue) [10]. Because of these difficulties, many patients with endometriosis are left either un-diagnosed, or take longer to get a final and correct diagnosis [8, 11]. Studies conducted in the USA and UK assessed the time elapsed from patients' pain complaint until they are diagnosed with endometriosis (via surgical confirmation). These studies revealed that women in USA experienced a means of 11.7 years from time of first symptoms to diagnosis of endometriosis, while women in the UK experienced a mean of 8 years [8, 11]. Although the time to diagnosis is significantly less in the UK compared to the USA, this time frame of approximately 8 years to diagnosis is unacceptable. In the US, it is estimated that incidence of the disease is higher in women 25-29 years of age, and lower in women older than 44 years [12].

There also appears to be some association, albeit be it weak, with race and endometriosis. Four studies have been conducted, one which suggests that Asian women are more susceptible to endometriosis compared to Caucasian [13]. The remaining studies suggest that endometriosis prevalence may be higher in Caucasian women compared to African-American women [14-16].

2. Endometriosis Classifications:

Endometriosis is classified into stages based upon the physical characteristics, the location, and invasiveness of the lesions [2]. Surgeons use the revised scoring system of the American Society for Reproductive Medicine (rASRM) to determine the endometriosis stage [17]. There are four endometriosis stages, defined by a point system, that are well- established by the ASRM (American Society for Reproductive Medicine). The total points are calculated, and the stage is determined accordingly. Staging is initially performed by measuring the number and the size of the endometriotic lesions in the pelvic cavity and determining their precise locations. The location and the extent of adhesions are also defined, and the maximum score is given to the peritoneal, ovarian, and cul-de sac lesions/regions. The identification of endometriosis in vagina, cervix, fallopian tube, intestine, and urinary tract and skin are added as in the “additional endometriosis” section [17]. Figure 2 shows the procedure used to determine the score based of endometriosis lesions in patients. The following list represents the stages of endometriosis [18]:

- Stage I (Minimal endometriosis): limited number of discrete flat superficial implants in pelvic cavity, signs of inflammation around the tissues, and minimal or no signs of adhesions (score 1-5 points) [17].
- Stage II (Mild endometriosis): superficial patches with adhesion and more inflammation (score of 6-15 points)[17].
- Stage III (Moderate endometriosis): The most recognizable sign of this stage is the development of endometriomas, which are red-brown cysts (chocolate cyst) on the ovaries, that grow with firm adhesion (score of 16-40) [17].

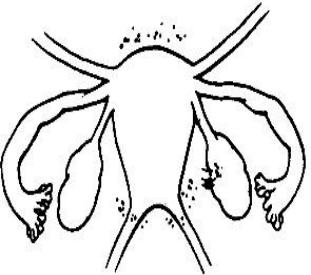
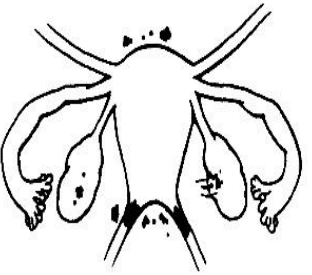
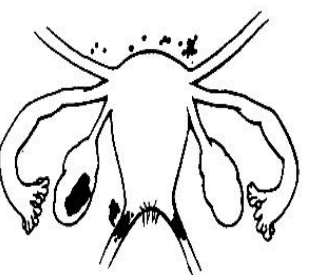
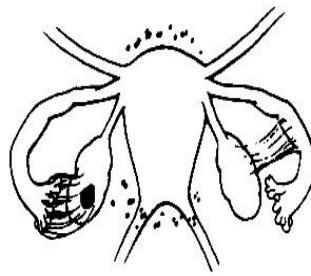
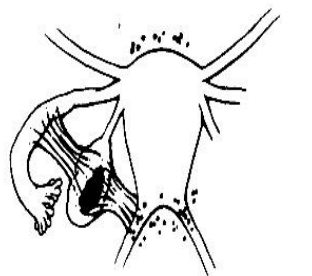
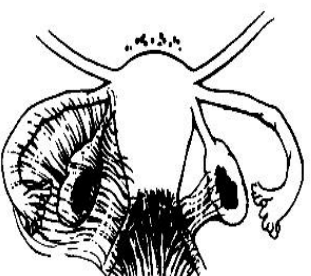
- Stage IV (severe endometriosis): This stage is characterized by an increase in the number and size of deep lesions and chocolate cysts that cause firmer adhesions (score of > 40 points)[17].

The goal of endometriosis staging is to provide uniform description of the lesions detected in laparoscopic diagnosis. However, the usefulness of this system is debated due to several issues. There is no correlation made between the characteristics of the lesions and the severity of the disease [19, 20]. It is well-known that the stage of endometriosis is irrelevant to the associated symptoms; a patient with stage one of endometriosis may experience more intense pain compared to a patient with stage four, who may have an asymptomatic form of the disease [20]. In addition, the system provides no suggestion to improve treatment options for patients with endometriosis [19, 20]. Although far from perfect, the rASRM classification system does allow some degree of standardization of endometriosis allowing consistency in classification among different physicians.

The Endometriosis Fertility Index (EFI) is another staging system developed to assess a patient's prospective fertility status after surgical removal of endometriotic lesions [21]. The EFI not only includes the rASRM components of endometriosis classification, but also provides a detailed score of the fallopian tubes/oviducts, the fimbriae and the ovaries. The EFI also expands beyond these scoring points to include conception-related factors such as age of patient, duration of infertility and gravidity history. However, the use of this system is also controversial because it focuses on the fertility aspect of the disease with no consideration to the major complaint in endometriosis patients; pelvic pain [8].

Figure 2. The guidelines for endometriosis stages and corresponding scores. (adapted from Revised American Society for Reproductive Medicine classification of endometriosis: 1996, Fertility and sterility 67(5) (1997) 817-21)[17]

Figure 2

EXAMPLES & GUIDELINES		
STAGE I (MINIMAL)	STAGE II (MILD)	STAGE III (MODERATE)
		
PERITONEUM Superficial Endo - 1-3cm - 2 R. OVARY Superficial Endo - < 1cm - 1 Filmy Adhesions - < 1/3 - 1 TOTAL POINTS - 4	PERITONEUM Deep Endo - > 3cm - 6 R. OVARY Superficial Endo - < 1cm - 1 Filmy Adhesions - < 1/3 - 1 L. OVARY Superficial Endo - < 1cm - 1 TOTAL POINTS - 9	PERITONEUM Deep Endo - > 3cm - 6 CULDESAC Partial Obliteration - 4 L. OVARY Deep Endo - 1-3cm - 16 TOTAL POINTS - 26
STAGE III (MODERATE)	STAGE IV (SEVERE)	STAGE IV (SEVERE)
		
PERITONEUM Superficial Endo - > 3cm - 4 R. TUBE Filmy Adhesions - < 1/3 - 1 R. OVARY Filmy Adhesions - < 1/3 - 1 L. TUBE Dense Adhesions - < 1/3 - 16* L. OVARY Deep Endo - < 1 cm - 4 Dense Adhesions - < 1/3 - 4 TOTAL POINTS - 30	PERITONEUM Superficial Endo - > 3cm - 4 L. OVARY Deep Endo - 1-3cm - 32** Dense Adhesions - < 1/3 - 8** L. TUBE Dense Adhesions - < 1/3 - 8** TOTAL POINTS - 52 *Point assignment changed to 16 **Point assignment doubled	PERITONEUM Deep Endo - > 3cm - 6 CULDESAC Complete Obliteration - 40 R. OVARY Deep Endo - 1-3cm - 16 Dense Adhesions - < 1/3 - 4 L. TUBE Dense Adhesions - > 2/3 - 16 L. OVARY Deep Endo - 1-3cm - 16 Dense Adhesions - > 2/3 - 16 TOTAL POINTS - 114

3. Symptoms:

The majority of women who suffer from endometriosis list chronic pelvic pain (CPP), dyspareunia (painful sexual intercourse), dysmenorrhea (painful menstruation), abnormal bleeding, and infertility as their chief complaints [4, 8, 22-24]. Endometriosis was confirmed laparoscopically in two thirds of adolescent girls with chronic pelvic pain or dysmenorrhea [25]. In the USA, it is documented that 79.1% of endometriosis patients may experience pelvic pain, 70.2% experience dysmenorrhea, and 49.5% complain of dyspareunia [26]. The associated pain is hypothesized to be a result of the estrogenic nature of the disease that increases pain sensitivity, bleeding of the extra-uterine lesions, and the ability of the lesions to produce cytokines that stimulate generation of nerve fibers, which in turn exacerbates the pain and inflammatory responses [27, 28].

Endometriosis patients may take longer to conceive (sub-fertile) or may be unable to conceive (infertile). In addition, the prevalence of endometriosis in infertile women is higher compared to fertile women [29]. The cause of these findings is not yet understood; however, it is speculated that infertility is a result of hormonal imbalance, peritoneal adhesions and extra-uterine endometrial lesions [30]. These factors induce changes in the pelvic anatomy and physically impair the ovary and fallopian tube functions and cause failure in releasing ovum or capturing it by fimbriae, or hindering its passage through the fallopian tube [30]. It is debated whether alteration in the physiology of the eutopic endometrium is a cause or a result of endometriosis [4]. It is hypothesized that these changes decrease endometrium receptivity, thus lowering implantation rates [4].

Several other conditions occur as comorbidities with endometriosis including irritable bowel syndrome. Common symptoms of both diseases include nausea, diarrhea, constipation and dysphasia due to the extrauterine lesions, which may cause bowel obstruction in rare cases [31].

In addition, the presence of endometriotic lesions on the bladder is reported to result in partial dysfunction of the urinary tract [6].

Studies have linked endometriosis with the onset of depression and anxiety [32]. This may be attributed to the chronic pain associated with the disease. A study was conducted to compare the psychological stress and depression in three groups: endometriosis patients with pain, pain-free patients, and a control group with no endometriosis. The study showed that the quality of life and the psychological conditions were compromised in patients who have endometriosis-associated with pelvic pain compared to the other two groups [33]. The anxiety and depression in endometriosis patients was not correlated with the stage of the lesion nor the age of the patients [34]. In addition, it is hypothesized that the mental disturbance in endometriosis patients may increase the intensity of the pelvic pain associated with endometriosis, which in turn increases the psychological stress [35].

4. Diagnosis:

The diagnosis of endometriosis is a daunting task. Laparoscopy is the gold standard method to detect the lesions [2, 17], but diagnostic laparoscopy is often delayed several years from symptom onset. One of the main reasons for the lack of non-invasive diagnostic tools is the lack of conclusive knowledge of disease pathophysiology and development. This leads to a delay in patient diagnosis considering that invasive method such as laparoscopy is a final step in evaluating patients who presented in gynecology offices with pelvic pain and infertility, alarming endometriosis symptoms [8]. The following approaches are used for the diagnosis of endometriosis:

4a. Physical exam and family history:

The initial criteria for suspected endometriosis are chronic pelvic pain, which is followed by obtaining the medical, social, and family history of the patient to rule out or include other conditions. In addition, pelvic examination is necessary to check for potential gynecological diseases, which feature soreness and focal pain. These characteristics are initiated in 97% of patients with pelvic pathology and 66% of endometriosis cases [36]. The similarities of these features with other gynecological and abdominal diseases complicate and interrupt the diagnosis. Depending on the patient complaints and physician experience, the following methods could be used:

4b. Surgical approach:

Laparoscopy or laparotomy are the absolute procedures to confirm endometriosis [17]. This is performed usually with the isolation of pathological specimens to confirm the presence of endometrial tissues; epithelial glands and stroma. In addition, staging of endometriosis is accomplished during surgery by following the revised scoring system of the American Society for Reproductive Medicine to assess the lesion location, characteristics, color, and invasiveness [2, 8, 17].

4c. Imaging approach:

There are three methods to detect some forms of endometriotic lesions by radiology, including; transvaginal ultrasonography, magnetic resonance imaging (MRI), and Doppler ultrasonography [37]. Yet, these provide very restricted access and visualization to a wider area of the pelvic cavity, which make them less-than-ideal to exclusively rely to diagnose the disease. Transvaginal ultrasonography and magnetic resonance imaging (MRI) have up to 90% specificity in detecting ovarian endometriomas, however, these methods deliver very little sensitivity in identifying

peritoneal and ovarian endometriotic lesions and associated adhesions. Likewise, Doppler ultrasonography, which estimates the speed and direction of the blood flow through blood vessel, provides little information about the presence of lesions. In endometrioma, the insufficient blood flow is considered as an indicator that requires further assessment [37].

Considering these limitations provided by imaging tests, surgical intervention is a necessity to acquire a definitive diagnosis of endometriosis.

5. Treatment and Therapeutic options:

Endometriosis is a chronic inflammatory disease; and no ultimate cure has been found so far. Thus, the available options provided for endometriosis is to alleviate the associated symptoms of pain and infertility.

5a. Pain management:

i. Pain Medication and hormonal therapy:

The objective of using a combination of pain medication and hormonal treatment is to control the pain in endometriosis patients before diagnostic laparoscopy. The primary approach of therapy is the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and combined oral contraceptive [2]. Due to the anti-inflammatory effect, NSAIDs such as naproxen are commonly used to decrease pelvic pain and dysmenorrhea. However, a recent randomized controlled trial demonstrated that there is no evidence in the robust effectiveness of NSAIDs in relieving pain in endometriosis patients compared to placebo. Additionally, the use of NSAIDs was shown to cause nausea, vomiting, headache and other side effects, which make it unfavorable choice to some patients [38].

Endometriosis treatment depends on the fact that endometriosis is an estrogen-dependent disease. Consequently, most of the available medications inhibit the production or the action of estrogen

such as oral contraceptives (OCPs), aromatase inhibitors, and GnRH analogues [39-43]. Alternatively, in severe stages of endometriosis when the available treatments fail to improve patient conditions, laparoscopic surgery is performed to remove the ectopic lesions. However, the surgical intervention is not the best approach for all patients especially for patients who have severe widespread endometriosis, because it may result in partial or complete hysterectomy to eradicate the disease. Controlling endometriosis through medications brings more hope for patients to improve quality of life and continue their reproductive life [44, 45].

Due to low cost and availability, combination estrogen/progestin contraceptives and progestogens are used as the primary treatment options for endometriosis patients. These hormones, such as medroxyprogesterone (DMPA) and ethinyl estradiol, act in inhibiting ovarian function and subsequently in alleviating the pain associated with endometriosis [46-48]. However, there are considerable undesirable side effects including preventing pregnancy, depression, nausea, and weight gain, which make contraceptives less-favorable for some patients [49]

Similarly, Dienogest (19-nortestosterone derivative) is a progestin oral contraceptive that is used for endometriosis treatment. Dienogest binds to PR (progesterone receptor) with high specificity and reduces the level of estrogen, has an anti-androgenic activity by acting as AR (Androgen receptor) antagonist, and reduces the inflammation and the proliferation of endometriotic cells [50, 51]. Because of these features, the side effects associated with Dienogest administration are considerably lower than other available medications [52].

Aromatase inhibitors (AI) are another treatment option for women with endometriosis. Aromatase is an enzyme that stimulates the conversion of steroidal precursors to estrogen, has been shown to be elevated in endometriotic lesion tissue, and as such, proposed to contribute to endogenous

production of estrogen by the lesion tissue. Therefore, inhibiting aromatase activity by AI reduces the level of endogenous as well as circulating estrogen levels which regresses endometriotic lesions and lessens associated pain [53]. Administration of this medication alone is linked to adverse effects. To limit these risks, it is recommended to use AI in a cocktail regime with other hormonal therapy such as OCPs or GnRH analogues for endometriosis treatment [54, 55].

Gonadotropin - releasing hormone (GnRH) analogs, which block the release of gonadotropin hormones and thereby reduce ovarian estrogen production, are a common treatment for endometriosis [42]. The reduction in estrogen level limits the growth and the survival of the ectopic lesion and suppresses the disease-associated symptoms. Yet, the hypo-estrogenic state introduced by GnRHa initiates some side effects such as reduced bone density, hot flashes, sweating, psychological disturbances; anxiety and depression. These associated outcomes arise because estrogen is necessary in various physiological processes other than uterine development, such as bone maintenance and cardiovascular health. As a result, “add-back” therapy is followed to avoid these symptoms. A recent comparative study showed that administration of GnRHa together with estradiol valerate and norethisterone acetate can mitigate the unfavorable effects and decrease the bone loss [56]. However, one must keep in mind that estrogen add-back therapy must be tailored to each patient to avoid “flare-up” of lesion growth and return of symptoms. Clearly, hormonal therapy is an important component of endometriosis treatment, but is far from ideal.

ii. Surgical approach:

If hormonal therapy fails in relieving pain, surgical intervention is introduced to control the symptoms. There are several surgical procedures suggested to manage endometriosis depending on the associated symptoms, including laparoscopic excision, laparoscopic presacral neurectomy (LPSN), and hysterectomy.

Laparoscopic excision of the lesions performed for patients who did not benefit from medications and continue to have pelvic pain including dysmenorrhea and dyspareunia. At one year after the surgery, 90% of patients documented improved symptoms after the laser laparoscopy. However, endometriosis has been shown to relapse in 30-50 % of patients five years after laparoscopy. Thus, many patients have to go through subsequent surgeries to control the disease [57] [58].

In patients with ovarian endometriomas (> 3 cm), drainage and ablation are proposed procedures; however, the ideal approach is laparoscopic excision to remove the cysts, control the pain, and improve fertility [59]. Nevertheless, there is a considerable chance of damaging the ovaries and reducing ovarian reserve. In general, there are some complications that may develop after laparoscopic surgeries such as adhesions development, intestinal and bladder injury, nerve injury, and gas embolism [60, 61].

Some patients show low, or no improvements after medical and surgical intervention. Thus, laparoscopic presacral neurectomy (LPSN) is performed which involves elimination of the nerve fibers around the interiliac triangle. It is considered a third line of treatment in these cases. Combination of ablation laparoscopy and laparoscopic presacral neurectomy (LPSN) is a more successful approach in reducing the pain and treating primary and secondary amenorrhea [62].

Hysterectomy that includes complete or partial removal of the uterus is considered a fourth line of treatment when all medical and surgical options have not achieved symptomatic relief. The procedure could be also performed in a combination with oophorectomy (removal of ovaries) and salpingectomy (fallopian tube removal) [63, 64]. Unfortunately, this aggressive approach is not successful in improving the symptoms in all patients. As a result, it is recommended to follow it with hormonal therapy (estrogen plus progesterone) to reduce the recurrence rate of the disease. It

is demonstrated that around 10% of patients re-develop pelvic pain after hysterectomy. This is attributed to the potential existence of ovarian remnants and endometriotic lesions, and to the type of hysterectomy performed [65, 66].

Due to the complications associated with the surgical interventions and the need for follow-up medicated regime afterward, the necessity for non-invasive options to treat the disease have been proposed in several studies.

5b. Infertility management:

Infertility in endometriosis arises from several hormonal and physiological disturbances caused by the growing lesions. In addition, the available treatment options to alleviate the pain aim to target the ovarian hormonal pathway, which creates another issue of pausing a woman's fertility. Thus, many endometriosis patients require medical assistance to conceive, which is achieved through medications and assisted reproductive technologies (ART).

6. Risk factors:

There are certain factors that are assumed to increase or decrease the risk of endometriosis. Most of these factors represent recommendations to maintain healthy life in general and not related to endometriosis only [10, 12].

6a. Physiological factors:

One of the most important factors is an early age at menarche, which is expected to increase the risk of endometriosis [12]. Early puberty and menstruation, in addition to obstruction of menstrual outflow, such as in Müllerian anomalies, impose a hormonal alteration that persists longer in those women. More importantly, the higher level of circulating estrogen initiates a favorable environment for the development of endometriotic implants, which rely on estrogen for growth

and survival [67]. There is also a positive association of endometriosis and lower BMI (Body Mass Index), which is demonstrated by higher tendency of lean women to develop the disease [10, 68-70]. Interestingly, the incidence of endometriosis is increased in women who were born at low birth weight [71]. Furthermore, short menstrual cycle is another factor that is expected to increase the risk of endometriosis [10, 70, 72].

6b. Pregnancy and breastfeeding:

Women who have given birth one or more times are at a lower risk of developing the disease compared to nulliparous women, which is attributed to increased level of circulating estrogen [16, 73]. Furthermore, breastfeeding was found to be associated with lower endometriosis susceptibility, which is explained by the postpartum amenorrhea and reduction in the level of gonadotropins [74] .

6c. Oral contraceptive:

Oral contraceptive use is associated with low risk of endometriosis. However, considering that endometriosis is controlled by hormonal oral contraceptives, it is not well-known if the lack of endometriosis symptoms in these women is due to the suppression of ectopic lesions, because of these medications, or due to the definitive free-disease state [75].

6d. Life style:

Following a healthy life style; consumption of fruits, vegetables, fish, and omega-3 fatty acids, in addition to regular exercise are associated with decrease risk of endometriosis [72, 76] . Exercise and omega-3 fatty acids are demonstrated to decrease the level of inflammatory markers including tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) [10, 77]. These two markers are

assumed to stimulate implant establishment. In contrast, consuming alcohol is linked to increased disease development [12, 78].

Likewise, cigarette smoking is suggested to reduce the incidence of endometriosis [72]. This surprising data might be explained by a study that demonstrated that the anti-estrogenic effects of nicotine [79, 80] may suppress lesion survival and manifestation of symptoms. Although the rationale behind this observation is reasonable, other groups revealed contradicting results and showed that cigarette smoking was not associated with the prevalence of the disease [81, 82].

6e. Genetic predisposition:

The involvement of a genetic factors in endometriosis development is debated. Yet, the available data suggest that there are genetic variants that need to be considered and investigated. Several studies demonstrated there is higher incidence between siblings and daughters of mothers diagnosed with endometriosis. In addition, cases of endometriosis in twins provided further evidence that supports the interplay between endometriosis and genetic make-up [83]. The difficulties of pointing out the potential genetic variant are due to the lack of large confirmed cases and the heterogeneity of the lesions. There are approximately 76 candidate genes that are expected to be associated with endometriosis [83]. Most of these genes are responsible for inflammatory disorders, and regulation of steroidal mechanisms. For example, interleukin 6 (IL-6), interleukin 10 (IL-10), signal transducer and activator of transcription 6 (STAT6), cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), progesterone receptor (PGR), tissue inhibitor of metalloproteinases-2 (TIMP2), and epidermal growth factor receptor (EGFR) have all been associated with endometriosis [83]. These data agree with the origin of endometriosis because it is a chronic inflammatory and estrogen-dependent disease. Furthermore, genetic linkage to chromosome 10 and chromosome 20 was associated with endometriosis development and found

to be significant in studies conducted among sisters and twins in Australian and UK research groups [84].

6f. Environmental factors:

The development of endometriosis due to various environmental stimulants is controversial. It is speculated that organochlorines play a role in endometriosis pathogenesis [85-88]. Organochlorines (known as dioxins) are a group of structural and functional related elements that are composed of chlorines as main structure. Examples of different types of organochlorines are pesticides such as DDT (dichlorodiphenyltrichloroethane) and industrial by-products such as polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) or furans [86, 89]. PCBs are used in industrial products such as transformers, oil-based paints, plastics, and appliances. Because of its features in providing firmness, electrical insulation, and resistance to flame, is used widely in manufacturing. However, the structure of dioxins contains a high number of chlorine atoms making it a source of toxicity in humans and in the environment. Thus, the usage of PCBs in manufacturing is banned in United States, still, the leaked remnants in air, food, or soil from old products is considered an environmental and health hazards [86, 90, 91]. Leaked PCBs are carcinogenic materials absorbed by skin and gastrointestinal and cause illness in the reproductive system, nervous system, endocrine system, and liver [92].

The contribution of dioxins in endometriosis development is based on the observation that dioxins may bind to estrogen and androgen receptors, promote the activity of estrogen, and consequently stimulate the growth of endometriotic lesions [87, 93, 94]. In addition, dioxins were found to cause mucosal immunity dysregulation which may enhance endometriosis development [95].

The level of dioxins, for instance PCBs, in endometriosis patients' samples was examined in several studies and found to be significant compared to control women, and the level was also associated with the stage of the lesion [96, 97]. However, other groups demonstrated that the concentration of organochlorine in serum of diseased and control women was insignificant [98, 99]. Indeed, these contradicting findings ignite the debate of whether dioxins are a promoter for endometriosis or not.

II. Endometriosis Pathogenesis:

The pathophysiology of endometriosis is not well-understood. Therefore, the availability of less-invasive diagnoses and more specific treatment options are limited. Several theories have been proposed to explain the origin of the disease. One of the first theories was based on the speculation that the ectopic endometrial tissue is transformed from normal peritoneal tissue; coelomic metaplasia [100]. The transformation of the tissues is explained to be caused by stimulatory factors, hormonal or immunological [101-104]. The metaplasia theory suggests the differentiation of bone marrow stem cells, mesenchymal stem and endothelial stem progenitors into endometriotic tissue [105]. Furthermore, it has been hypothesized that endometriosis is developed as a result of benign metastasis which originates from the circulation of the endometrial cells in blood and lymphatic systems [106]. Immunity imbalance is expected to cause the development of the ectopic endometrial lesions because of the statistics that show that autoimmune disease is more prevalent in women with endometriosis [101, 107-109]. Although these theories may provide insight into the origin of the disease, the mechanism of the migrating, attachment, and surviving of endometriotic lesions ectopically is not well-explained. Thus, evidence to support these theories and investigate pathways of lesion establishment are examined by studying the differential expressed proteins and mRNAs, in women with endometriosis compared to endometriosis-free

women. Theories of endometriosis development, and the transcriptomes and proteomic alterations are explained in detail below:

1. Retrograde Menstruation:

Retrograde menstruation is the most widely accepted theory for the development of endometriosis [14]. According to this theory, during menses, a portion of the endometrial lining is also shed retrogradely through the fallopian tubes into the peritoneal cavity. Retrogradely shed endometrial fragments attach in the pelvic cavity leading to the implantation and development of endometriotic lesions [14]. It is estimated that around 70%-90% of women of reproductive age encounter retrograde menstruation, however, only a few (approximately 10%) of these develop endometriosis [110]. This theory is supported by the existence of endometrial debris in the pelvic cavity of patients with endometriosis compared to healthy women and by the reduction of endometriotic lesions in women who were treated by endometrial ablation that involves destruction of the endometrium [111]. Moreover, endometriosis induction was established in non-human primates, baboons and macaques, which develop spontaneous endometriosis through inoculation of endometrial tissue into the pelvic cavity. The development of the ectopic endometriotic tissues, which are morphologically similar to human endometriotic lesions, are successful in 40% of the baboon model after one inoculation, and in 100% of the baboons after double inoculations. [112, 113]. Donnez and colleagues [114] induced the two layers of the endometrium (functionalis and basalis) ectopically in the animal model, which leads to the development of deep nodular endometriosis. The regeneration of endometrium is thought to be from stem cells located in the basalis; however, only the upper layer of functionalis sheds during menstruation. Therefore, this model may not recapitulate the actual process of retrograde menstruation. In women with cryptomenorrhea, a congenital obstruction of the vagina that causes menstrual flow to be retained

internally, the risk of endometriosis is increased which is attributed to the larger amounts of sloughed endometrial tissues to the peritoneal cavity, which is an indication of the rationality of the retrograde menstruation theory [100].

Although the theory of retrograde menstruation is widely supported, it doesn't explain observations of endometriosis in men and pre-pubertal girls [110, 115, 116]. Also, it doesn't provide explanations of the factors that make these women more susceptible to develop the disease and promote lesion survival and proliferation. If this theory is true, we still have several gaps to fill to understand exactly how the migrated ectopic lesions escape the "immune-surveillance" system, and what mechanisms contribute to the growth, angiogenesis, and establishment of the implants.

2. Metaplasia:

The metaplasia theory proposes that endometriosis is established because of transformation of normal epithelial cells or undifferentiated cells into ectopic endometriotic lesions, and this is represented by coelomic metaplasia, and trans-differentiation of Wolffian or Mullerian ducts in female embryos [117]. Coelomic metaplasia is the process of transformation of celomic epithelia cells that line the abdominal organ and abdominal wall into endometriotic lesions, under the influence of estrogenic and inflammatory stimulants [117]. Trans-differentiation of Wolffian or Mullerian ducts theory suggests that the endometrial cells outside of the uterus are generated originally from imperfect growth of Wolffian or Müllerian ducts during embryonic development. This theory speculated that ectopic endometrial lesions are developed as a result of Wolffian or Mullerian duct remnants which progress into estrogen-responsive lesions [100]. Although these theories may provide some explanations to the establishment of endometriosis in pre-pubertal girls [115], the amount of estrogen at this age is very low to stimulate the growth of ectopic lesions. In

addition, the observations of endometriotic implants outside of the pelvic cavity was not taken into consideration.

3. Immunity dysfunction:

The theory of immunity dysfunction was developed due to the observation that women with endometriosis show high levels of activated macrophages, and decreased activity of natural killer cells [103, 118]. According to this theory, the buildup of the endometrial tissues in the peritoneum stimulates the inflammatory response, leading to the local activation of macrophages and leukocytes [103, 118]. However, due to the defective immunity, the recruited inflammatory factors fail to be recognized by the immune response. This leads to the growth and establishment of the ectopic lesions causing endometriosis [101].

4. Stem cells:

Stem cells are undifferentiated cells that are capable of self-renewal and differentiation into certain types of cells under specific stimuli. The development of endometriosis as a result of stem cell translocation to the perirenal cavity is supported by the evidence of the presence of fractions of basal layer in the ectopic lesions. Basal layer consists of clonogenic cells that are the source of endometrial tissue renewal, however, this layer doesn't shed during menstruation [1]. The observation of the existence of clonogenic cells and endometrial cells in ectopic lesions provides evidence of a potential role of stem cells in endometriosis development [119]. Based on these observations, it is hypothesized that during menstruation, a portion of basal layer is also shed with the endometrial tissue retrogradely, and the stem cell components can differentiate into endometriotic lesions [120]. Supporting this theory, endometriotic lesions in baboons were successfully established in 100% of the animals through implantation of a pool of endometrial tissues and basal layer [121]. Although this observation provides strong evidence of its validity,

it doesn't provide an explanation for the regeneration of endometriotic lesions in locations other than pelvic cavity. Therefore, the migration of bone marrow stem cells through the lymphatic system to remote sites was suggested to fill this gap [121, 122].

5. Transcriptome and proteomes alterations:

Changes in mRNA and protein levels in eutopic endometrium, ectopic lesions, peritoneal fluid, and serum of endometriosis patients are hypothesized to be a cause of endometriosis [123]. Yet, several studies suggested that these alterations may develop as a result of the disease [124]. Thus, the findings of these observations are proposed to provide diagnostic biomarkers and/or therapeutic targets. On the transcriptome level, the deregulation of miRNAs has also been investigated as a cause or result of endometriosis [125-131].

5a. miRNAs:

miRNAs are short non-coding nucleotide sequences that regulate the expression of target genes [132, 133]. The biogenesis of miRNA transcript is initiated in the nucleus under the direction of polymerase II or III to form the long primary RNA transcript (pri-miRNA)[134]. The capped and poly-adenylated pri-miRNA forms a complex by binding to the RNA-binding protein DGCR8 (DiGeorge syndrome critical region 8) [135-137]. This is followed by the action of Drosha (an RNase III enzyme) that cleaves pri-miRNA–DGCR8 complex to make pre-miRNA, a stem-loop precursor [138, 139]. The pre-miRNA is transported into the cytoplasm and undergoes under another cleavage by DICER, RNase III enzyme, which cleaves the pre-miRNA into an RNA duplex [140]. The generated strands of the miRNA duplex go through different pathways; one strand is degraded and the other strand binds to RISC, the RNA-induced silencing complex [140]. This creates the mature miRNA which recruited RISC to suppress gene transcription by binding to the 3'UTR of target and causes mRNA degradation or translation inhibition [141, 142]. miRNAs

may also induce repression by binding to the coding sequence or the 5'UTR of gene targets, a less common mechanism [143, 144]. Of all miRNAs, the only exception to this pathway is miR-451 (now known as miR-451a), which is cleaved into RNA duplex by Ago2 protein and continues to form mature miR-451a, independently of DICER action [145-147]. Figure 3, represents the two pathways of miRNA biogenesis, a canonical pathway of miR-144 and Dicer-independent pathway of miR-451a [147].

miRNAs are essential in the normal development of tissues, thus, alteration in expression is associated with several diseases including cancer, inflammatory and auto-immune diseases [148-151]. In endometriosis, there are several studies that hypothesized the role of miRNA in the pathophysiology of endometriosis [125-131]. The expression profile of mis-expressed miRNAs in endometriotic lesions, eutopic tissues, and eutopic endometrium from disease-free women has been reported by several research groups [152-156]. One of the most differentially expressed miRNA is miR-451a, which was shown by microarray analysis to be two-fold lower in endometriosis samples (eutopic endometrium and ectopic lesions) compared to endometrial tissues from women without endometriosis [152]. Further studies using next-generation sequencing technology demonstrated that miR-451a is up-regulated in ovarian endometrioma compared to eutopic endometrium [154]. It is proposed that miR-451a acts as a tumor suppressor and reduces cell survival by targeting genes responsible for cellular proliferation, apoptosis, and invasion [131, 157-162]. Evidence of this role was established in endometriosis using bioinformatic prediction tools, PCR, western blot, and reporter assay [131, 157-162].

Previously in our laboratory, miR-451a was demonstrated to be elevated in endometriotic lesions and reduced cell proliferation by directly binding to the 3'UTR of MIF (macrophage inhibitory factor) and inhibiting its transcription [159]. MIF was originally identified as a potent mitogenic

factor for human endothelial cells in vitro and tumor angiogenesis in vivo [163]. Yang and colleagues demonstrated that, in patients with endometriosis, MIF could stimulate endothelial cell proliferation [164]. Further supporting a role of MIF in endometriotic lesion survival, MIF has been shown to stimulate PGE2, COX-236, vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and monocyte chemotactic protein-1 (MCP-1) expression [165], as well as the induction of aromatase expression in a feed-forward mechanism [166]. Interestingly, MIF also stimulates TNF- α secretion, whereas TNF- α is also capable of inducing MIF production in endometrial cells [167]. Also, of relevance to the pathophysiology of endometriosis is the demonstration that many of these MIF-induced factors are associated with a proliferative and angiogenic phenotype conducive to endometriotic establishment or growth (or both) [165].

Furthermore, Joshi and colleagues induced endometriosis in baboons and found reduced expression of miR-451a in the baboon's eutopic endometrium [131]. In the endometriotic epithelial 12Z cell line, miR-451a was found to directly target and repress the YWHAZ gene [131], which codes for 14.3.3 ζ (zeta) protein, causing a reduction in cell survival and proliferation [131]. 14.3.3 ζ protein plays a role in signal transduction and it regulates tumorigenesis and apoptosis [168, 169].

Preliminary data from our laboratory demonstrated that RPLP1, ribosomal protein large P1, may serve as a potential target of miR-451a. RPLP1, ribosomal phosphoprotein large P1, is part of the 60S ribosomal subunit. The RP (ribosomal proteins) composed of one RPLP0 and two heterodimers of RPLPL1 and RPLP2 that form the ribosomal stalk (Figure 4)[170-173]. P1 and P2 share similar molecular mass of 12 kDa and form a structure that play a role in translation process by interacting with the eukaryotic elongation factor, eEF-2 [174-176]. During the

elongation step of protein synthesis, the phosphorylated forms of P1 and P2 promote the GTPase activity of eEF-2 which stimulates the translocation of peptidyl-tRNA from the A to the P site [177]. P1 and P2 were also detected to be free in the cytoplasm and it speculated that an exchange occurs between the two status (free and ribosome bound) to modulate the translation process [176]. Although most of the research on ribosomal function of RPLP1 focus on its role in translation, few studies have evaluated additional functions. RPLP1 has been speculated to play a role in tumorigenesis. RPLP1 is highly expressed in gynecological tumors and stimulates cellular transformation [178]. In some ovarian cancers, the level of RPLP transcripts was found to be related to the p53 levels [178, 179]. Additionally, a study was conducted to examine the RPLP1 mechanism responsible for cell development and growth, which demonstrated that downregulation of RPLP1 caused endoplasmic reticulum (ER) stress and promotes autophagy [180]. Furthermore, Compao and colleagues found that RPLP1 and RPLP2 are essential host factors for replication of flavivirus, dengue viruses (DENV), yellow fever virus (YFV), and Zika virus (ZIKV) [181]. This study demonstrated that these viruses utilize both host proteins, RPLP1 and RPLP2, for viral protein translation, an important step to replicate inside human cells.

With respect to the role of RPLP1 in endometriosis, we are not aware of previous studies that correlate RPLP1 expression with that of endometriosis. However, the dysregulation of ribosomal RNAs and proteins expression in endometriosis has been reported [182-186]. Specifically, ribosomal protein L26 was one of the 91 up-regulated genes detected in eutopic endometrium from endometriosis patients compared to endometrium from women without endometriosis [185]. Additionally, expression of ribosomal protein S14 (RPS14) and ribosomal protein S9 (RPS9) is elevated in endometriotic lesions taken from patients with endometriosis versus endometrium from disease-free women [186]. An upregulation of around 10 ribosomal proteins, in endometriotic

lesions compared to paired endometrium tissues, was also found in a study conducted by Wei-Ping Hu and colleagues [184] and included ribosomal protein L7 (RPL7), ribosomal protein S17 (RPS17), ribosomal protein L9 (RPL9), ribosomal protein S4, X-linked (RPS4X), ribosomal protein S25 (RPS25), ribosomal protein L10a (RPL10A), ribosomal protein L41 (RPL41), and ribosomal protein L13a (RPL13A) [184]. Defects in ribosome biogenesis was suggested to promote endometriotic lesions development because of mis-expression of ribosomal proteins in endometrial tissues of patients with endometriosis [183]. Outside of these descriptive data, no mechanistic studies have been performed to evaluate a potential functional role of ribosomal proteins in the pathophysiology of endometriosis.

Figure 3. Representation of the miRNA canonical pathway biogenesis and miR-451 dicer-independent pathway. (Adapted from A. Dueck, G. Meister, MicroRNA processing without Dicer, Genome Biol 11(6) (2010) 123)[147].

Figure 3

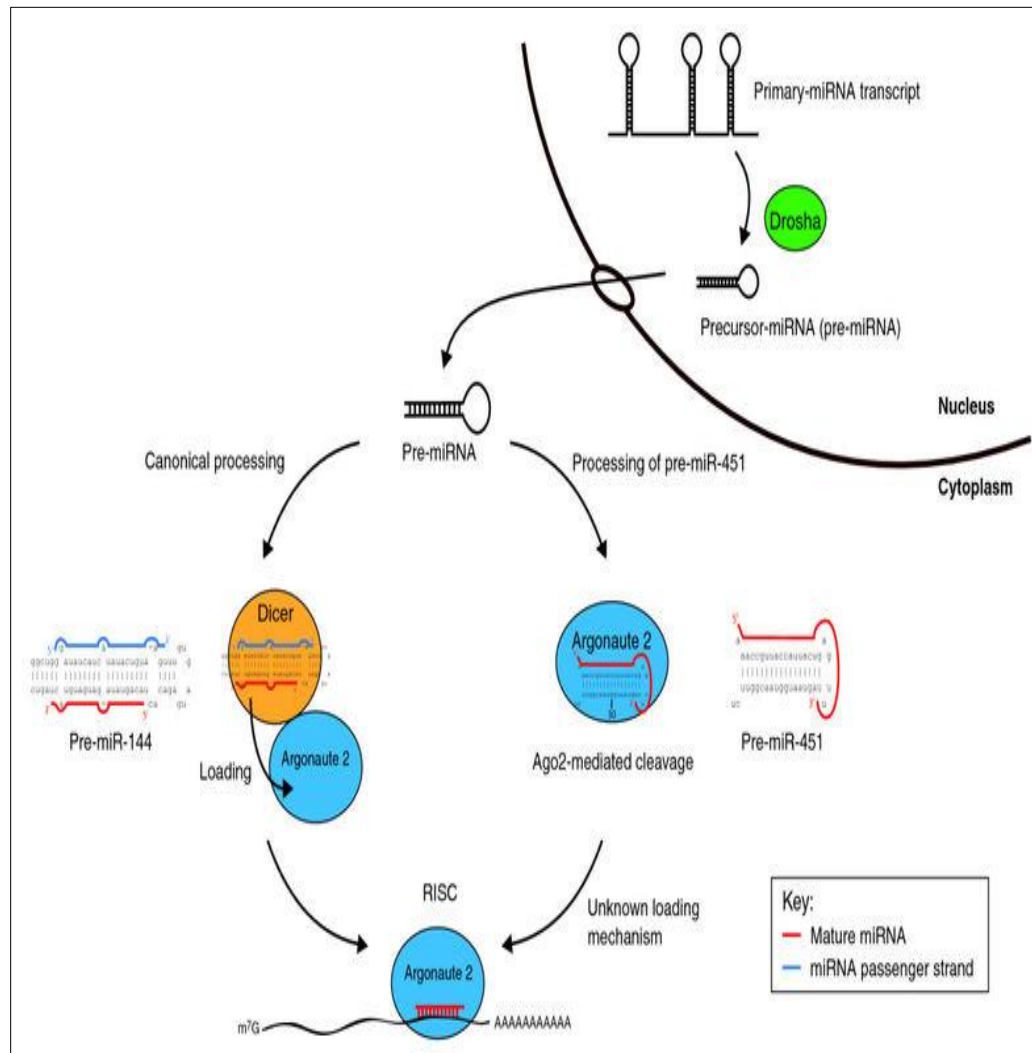
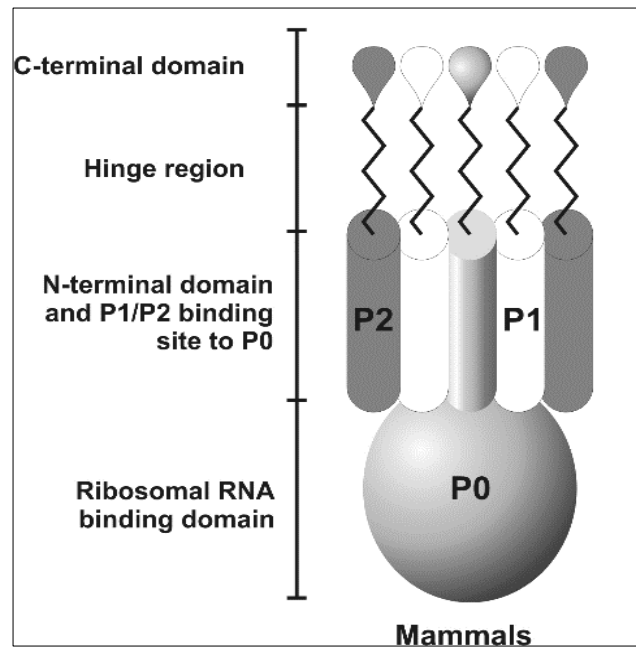


Figure 4. The interaction of ribosomal proteins P1, P2, and P0 to form the ribosomal stalk.
(Adapted from W.J.L. Maximiliano Juri Ayub, Johan Hoebeke and Cristian R. Smulski Ribosomes from Trypanosomatids: Unique Structural and Functional Properties Cell-Free Protein Synthesis (2012) 3-28) [173].

Figure 4



Objective of the research:

Endometriosis is an estrogen – dependent, debilitating disease, defined as the development of ectopic endometrial tissue, which establishes primarily in the pelvic cavity. The primary symptoms of the disease are pelvic pain and infertility [10]. Endometriosis is speculated to develop by retrograde menstruation in which endometrial fragments travel retrogradely through the fallopian tubes[14]. However, because essentially all women exhibit some degree of reverse menses, some other factors must lead to endometriosis in the subset of women who develop this disease [14]. The uncertainties about the development, as well as the establishment and progression of the disease have led to an inadequate ability to effectively treat the disease. One well-established fact is that estrogen is essential for endometriotic lesion survival. Thus, current treatment options target the suppression of the estrogen responsiveness of the tissue, which reduces the burden of the disease [187] . While suppression of estrogen action and or production is effective in many women, it is associated with unwanted and potentially detrimental negative side effects [28, 188]. Collectively, these shortcomings highlight the necessity for a better understanding on the pathogenesis of endometriosis so that more specific treatments with fewer side effects can be developed. Initial studies from our laboratory demonstrated that miR-451a plays a role in endometriosis pathogenesis through regulating the expression of several gene targets including RPLP1, Ribosomal Protein Large P1. Furthermore, we found that RPLP1 is highly expressed in the ectopic endometriotic lesions compared to the eutopic tissues. RPLP1 is a ribosomal protein that contributes to cellular proliferation, transformation, and tumorigenesis [178, 179, 189]. As this protein has been proposed to play a pro-survival role in diseases associated with enhanced tissue survival and proliferation, we propose that the elevated expression of RPLP1 in endometriotic lesion tissue may play a conducive role in the survival of ectopic lesion tissue. The

overall objective of our research is to examine the function and the expression of RPLP1 in endometriotic lesion tissue as well as its relationship with, and potential regulation by miR-451a. The following study is the first to identify the differential expression of RPLP1 in endometriotic lesions compared to matched eutopic endometrium as well as with endometrium from control non-endometriosis patients. The outcomes from this study have significant potential to deepen our understanding on critical modulators of endometriotic lesion survival which ultimately may translate into novel treatments for endometriosis which minimize or eliminate unwanted side effects. Additionally, development of novel approaches for endometriosis treatment which exhibit minimum alteration to the estrogen pathway will reduce the occurrence of the menopause like symptoms associated with current endometriosis therapy.

Chapter II:

RPLP1 Is a Novel Target of *miR-451a* Whose Expression Is Elevated in Endometriotic Lesion Tissue and Correlates with Endometriotic Lesion Tissue and Cell Proliferation.

Abstract

INTRODUCTION: Endometriosis is a disease common in women of reproductive age where endometrial tissue establishes and survives in ectopic locations. However, how these lesions establish and survive is unknown. We have recently identified ribosomal protein large P1 (RPLP1; which is a modulator of cell proliferation) as a potential target of miR-451a expressed in endometriotic epithelial 12Z cells. The objective of the current study was to determine the expression of RPLP1 in human endometriotic lesion tissue, examine the function of RPLP1 in modulating cell survival/proliferation and miR-451a regulation of RPLP1 expression.

METHODS: RPLP1 mRNA and protein levels were examined in paired endometriotic lesion tissue and eutopic endometrium from women with stage III/IV endometriosis (N=55) as well as tissue from women without symptoms of endometriosis (controls; N=30 for localization studies).

RESULTS: Net expression of RPLP1 mRNA was significantly higher in ectopic lesion tissue compared to paired eutopic endometrium (3.72-fold increase; $P < 0.01$) and immunohistochemical localization revealed predominant localization to epithelial cells (lesion > endometriosis eutopic > control eutopic; $P < 0.05$). As RPLP1 is proposed to drive cellular proliferation, we assessed the correlation between lesion RPLP1 mRNA expression and that of cyclin E1 and observed a positive correlation between both markers (Pearson $r = 0.571$; $P < 0.01$; N=69). To further demonstrate functionality, we generated a stable 12Z cell line in which RPLP1 was deleted and found that loss of RPLP1 expression was associated with a 90% reduction in cell number/survival ($P < 0.001$; N=3). We further demonstrated that over-expression of miR-451a via transient transfection of 12Z cells resulted in a significant ($P < 0.05$; N=3) reduction in RPLP1 expression as well as that of cyclin E1.

CONCLUSIONS: These studies reveal that RPLP1 is a novel target of miR-451a and that RPLP1 expression and its regulation by miR-451a are associated with cell proliferation and survival. These results, as well as our previous and ongoing studies, continue to support a role for miR-451a in modulation of endometriotic lesion tissue/cell survival which may prove useful as a non-hormonal therapeutic agent.

Introduction:

Endometriosis is a chronic inflammatory disease, characterized by the development of ectopic endometrial tissue, which establishes primarily in the pelvic cavity. The primary symptoms of the disease are pelvic pain and infertility [100]. Endometriosis is thought to develop through “seeding” of the pelvic cavity via retrograde menstruation of shed endometrial tissue during menses [14]. The mechanism that induces the migration, attachment, and survival of these lesions is not well-understood. However, several theories have been proposed to explain the origin of the disease.

One of the potential mechanisms for the development and/or survival of endometriotic lesions tissue was based on microarray analysis of ectopic lesions and eutopic tissues from endometriosis patients and women without endometriosis [152]. The result of the study illustrated several miRNAs that are differentially expressed in these tissues that may contribute to endometriosis pathogenesis. MiRNAs are conserved short nucleotides sequences that regulate the expression of their target genes and play a dynamic role in many biological and pathological processes [141]. In endometriosis, miRNAs have been demonstrated to control the expression of genes responsible of proliferation, cellular migration, and apoptosis. Above all, miR-451a has been recognized as one of the most mis-expressed miRNA between eutopic tissue and ectopic lesion of patients with endometriosis and eutopic tissue from disease-free women [152]. The level of miR-451a was speculated to be negatively associated with ectopic lesion survival and growth through modulating the expression of certain genes; MIF [128], fibrinogen alpha chain [128], and YWHAZ [159]. In a baboon model, induction of endometriosis was found to decrease the expression of miR-451a at 3 months post-induction and this was correlated with the elevation of its target gene, YWHAZ. Furthermore, immunohistochemical localization studies performed by this same group revealed

that miR-451a expression is mainly in the epithelium (glandular **and** luminal) compared to the stroma [131].

While the expression of miR-451a in human endometriotic lesion was confirmed, the exact mechanism by which miR-451a might reduce lesion aggressiveness is still uncertain. As such, the objective of the current study was to investigate the role of miR-451a in endometriosis. To do so, we began by screening differential proteins expression in endometriotic epithelial 12Z cells in which miR-451a was over-expressed. From the dozens of potential targets of miR-451a, we initially focused on RPLP1 (Ribosomal Protein Large P1). We selected RPLP1 as this protein has been associated with endometrial cancer [179]; a disease which shares similar characteristics with endometriosis in which cell migration, attachment and survival is enhanced. For the current study, we focused on evaluating RPLP1 mRNA and protein expression, examining its regulation by miR-451a and its function in human endometriotic lesion tissue and cells.

Materials and Methods

Cell culture and transfection for miR-451a assessment

For our in vitro studies, we used the well-characterized endometriotic epithelial cell line, 12Z (159) which were obtained from Dr. Linda Griffith, MIT via a Materials Transfer Agreement (MTA).

12Z cells were seeded at density of 1×10^6 cells/ml of media in T-75 flask using complete culture media: phenol red-free Dulbecco's Minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co.) + 10% charcoal stripped FBS (Atlanta Biologicals, Atlanta, GA, USA) + Pen-Strep (Sigma Chemical Co) for maintenance. Once the cells reached approximately 90% confluency, the cells were passed and plated in 6 wells plates at a density of 1×10^5 cells/ml in phenol red-free Dulbecco's Minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co.) with no FBS and P/S added and placed in the incubator for 24h at 37C and 5% CO₂.

For transfection assays: After 24h of plating the cell, 12Z cells were transfected using Lipofectamine 2000 transfection agent (Life Technologies/Ambion, Inc.) following the recommendations of the manufacturer with either Lipofectamine 2000 transfection reagent mixed with 30 nM of pre-miR451 (pre451; Life Technologies/Ambion, Inc., Grand Island, NY, USA) or a non-targeting mimic (NT-mimic, negative control; Life Technologies/Ambion, Inc.). At 24h and 48h post-transfection, RNA and proteins were collected for qRT-PCR, western blot, and 2D-gel analysis.

For RPLP1 transient knockdown, siRNA (small interfering RNA; Dharmacon: ON-TARGET *plus smart pool*) for RPLP1 was used. To prepare the cells for transfection, 12Z cells were plated

in phenol red-free DMEM: F12 containing 10% non- charcoal stripped FBS, 1% Pen-strep. As a control, non-targeting NT siRNA cDNA was used. For RPLP1 knockdown, Cells were transfected using siPORT™ NeoFX™ transfection agent (Life Technologies/Ambion, Inc.) following the recommendations of the manufacturer. Briefly, transfection reagent was mixed with 50 nM siRNA cDNA for RPLP1 and added to 6-well plates (.2ml volume). Then, the 12 Z cells (1×10^5 cells in 1.8 ml of complete media) was added to the plates and cultured for 24, 48, and 72h. For each treatment, cells were cultured in duplicates and then processed for proliferation/survival assessment, and for protein analysis. After each time point, the medium was removed and the number (percentage) of the dead cells was evaluated. The live cells (viable) were collected by mild trypsin digestion and then trypan blue dye was added to estimate the cells number and viability. All assessments were performed in duplicate and this experiment was repeated at least 4 times using different passages of the 12Z cells.

For stable knockdown of RPLP1 in endometrial 12Z cells: To develop stable RPLP1 deficient cell lines, 12Z cells were plated at 1×10^5 cells/well of 6-well plates in DMEM:F12 with pen/strep and 5% FBS for 24h. Cells were transduced with either lentiviral particles expressing a shRNA for RPLP1, or a non-targeting shRNA (negative control) per the manufacturer's instructions (GeneCopoeia, Inc., Rockville, MD). Cells were then cultured with puromycin (2 μ g/mL) to select positively infected cells. These stably transfected cells were maintained for experimentation or stored in liquid nitrogen for future experiments.

2D gel analysis

2D-DIGE (2-dimensional differential in-gel electrophoresis) was performed by Applied Biomix (Hayward, CA). Total protein was extracted from the 12Z cells transfected with either pre-miR-

451a or pre-NT transfected (as described above) using lysis buffer (Cell Signaling Technology, 9803) and .5% PMSF. For 2D-DIGE and Mass spectrometry, the extracted Protein samples were sent to Applied Biomics (Hayward, California). The experiment was performed using SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis by Typhoon TRIO (GE Healthcare). The image was scanned using by Image QuantTL 8.1 software (GE Healthcare-Biosciences, Pittsburgh, Pennsylvania) and analyzed (in-gel and cross-gel analysis) using DeCyder software version 6.5 (GE Healthcare) to identify the differential expressed proteins. The ratio changes of the protein differential expression between pre-miR-451a and pre-NT transfected cells in which protein expression was >1.5-fold were selected (25 protein total). Proteins were then isolated using Ettan Spot Picker (GE Healthcare) and subsequent MALDI-TOF (MS) and TOF/TOF (tandem MS/MS) using a 5800-mass spectrometer (AB Sciex).

For protein identification, the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer version 3.5 equipped with MASCOT search engine (Matrix Science) to search the database of National Center for Biotechnology Information non-redundant (NCBI nr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant. All proteins reported in this work had a C.I.% of 100%.

RNA isolation and qPCR

For assessment of mRNA, total RNA was isolated using the Tri-reagent (Sigma Chemical Co., St. Louis, MO, USA). Then, the total RNA (1 µg in 20 µl) was used for reverse transcription using

RT kits (Applied Biosystems; Foster City, CA, USA), according to the manufacture instructions. Primer-Blast was used for primers design, and for 18S (control). The primer used in this study are RPLP1 V1: forward 5'-TGACAGTCACGGAGGATAAGA-3', reverse 5'-CCAGGCCAAAAAGGCTCAAC-3'. RPLP1 V2: forward 5'-CTCACTTCATCCGGCGACTA-3', reverse 5'-GCCAGGGCCGTGACTGT-3'. CCNE1: forward 5'-CAGGGAGCGGGATGCG-3', reverse 5'-GGTCACGTTTGCCTTCCTCT-3'. PTEN: forward 5'-AAGACATTATGACACCGCCAAA-3', reverse 5'-GTGGGTTATGGTCTTCAAAGGA-3'. The primers were synthesized by Integrated DNA Technology (IDT, Coralville, IA). The resulting samples were used for qRT-PCR by using QuantStudio 7 Flex System. All samples were run in triplicate, and the calculation was done by using the average values, and the 2-delta-delta CT method. In human tissues experiment analysis, human 18S primers probe reagents were analyzed, and the fold changes were normalized to the eutopic endometrium. In 12Z human endometriotic cells experiment analysis, human 18S primers probe reagents were analyzed, and the fold changes were normalized to the non-targeting (negative control).

For miR-451a qRT-PCR analysis, total RNA of (250 ng in 5 µl) was reverse transcribed using miR-451a Kit (Applied Biosystems) and RT kits (Applied Biosystems) according to manufacture protocol. An individual reverse transcription was performed using 2 ul from each miRNA 5X RT primers. Using the resulting material, individual qRT-PCR for each specific miRNA using QuantStudio 7 Flex System. All Samples were tested in triplicates and normalized to U58 (control) to calculate the delta ct value. All data are represented as the mean \pm

Protein Isolation and Western blot:

The human tissues were homogenized in RIPA Buffer (Cell Signaling Technology, 9806) with PMSF (Cell Signaling Technology, 8553S) and total protein concentration measured using Bio-Rad by Protein Assays ([Catalog 3500-0006], Bio-Rad Laboratories, Richmond, CA, USA).

To isolate the protein from the 12Z human endometriotic epithelial cell line, the protein was isolated by using lysis buffer (Cell Signaling Technology, 9803) with PMSF (Cell Signaling Technology, 8553S), and then the Bio-Rad Protein Assay ([Catalog 3500-0006], Bio-Rad Laboratories, Richmond, CA, USA) was used to determine the total protein.

The same amount of protein (50 µg) was electrophoresed through 12% Bis (2-hydroxyethyl) amino-tris(hydroxymethyl)methane (w/v) gels and electro-blotted onto nitrocellulose membrane (Invitrogen). Primary antibody (RPLP1 1:500; ProteinTech 216363-I-AP) and goat anti-rabbit secondary antibody (1:5000; GE Healthcare/Fisher Scientific, Pittsburgh, PA, USA) were used. To normalize the protein expression levels, stripping and re-probing for beta-actin (ab8227) (Abcam, Cambridge, MA, USA). The immuno-detection was performed by using enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA).

Human Tissues

We utilized tissue samples from our IRB-approved bank of specimens which were obtained from the University of Kansas Medical Center, Department of Pathology and Laboratory Medicine and/or the Department of Obstetrics and Gynecology at the Cleveland Clinic. This consists of paraffin-embedded tissue sections for immunohistochemistry studies as well as frozen tissue for protein and RNA analysis (western blot and PCR, respectively).

Samples were obtained from subjects between the ages of 21 and 45 who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue. A total of 55 subjects were enrolled (N = 23 in the proliferative stage of the menstrual cycle, N = 32 in the secretory stage of the menstrual cycle and included women with stage I/II (N = 20) and stage III/IV (N = 35) endometriosis. No subjects had taken GnRH analogs or hormonal therapy within 3 months prior to surgery. A total of 55 endometrial biopsies (eutopic endometrium) and 77 matched (same patient) endometriotic lesions were collected. All specimens were collected by the same surgeon (TF) at Cleveland Clinic or University of Kansas Medical Center (KS) with emphasis on minimizing sample contamination from underlying/surrounding non-endometriotic lesion tissue. To do so, endometriotic lesions were excised and sent to pathology for confirmation of endometriosis, which was defined as the presence of endometrial glands and stroma. Tissue was excised using sharp scissors with no energy. During the excision the underlying tissue was separated from the lesion tissue. A portion of the same sample lesion which was sent for endometriosis confirmation by a pathologist was utilized for research. Research samples were immediately snap-frozen, stored at -80 °C and then shipped to the University of Kansas Medical Center. Stage of the menstrual cycle was determined from the patient's medical records with day 1 defined as the onset of menses.

Tissue samples used as non-endometriosis controls were obtained from the University of Kansas Medical Center Department of Pathology and Laboratory Medicine. Endometrial tissue in the control groups consisted of women with uterine leiomyomas (N=12) or endometrial polyps (N=8) of which 10 samples were obtained during the proliferative stage of the menstrual cycle (N=10) and ten from the secretory (N=10) stage of the menstrual cycle. Eutopic and matched ectopic endometriotic lesion tissue (ectopic) were obtained from women with endometriosis during the

proliferative (N=12; N=4 stage I/II and N=8 stage III/IV) and secretory (N=12; N=6 stage I/II and N=6 stage III/IV) stage of the menstrual cycle. As no difference in RPLP1 mRNA, protein or miR-451 expression was noted among stages of the menstrual cycle or stages of endometriosis, data were collapsed and analyzed as ectopic versus eutopic tissue unless otherwise noted.

Immunohistochemistry staining and histo-scoring

The tissues were fixed with 10% neutral buffered formalin and subjected to immunohistochemical (IHC) localization using RPLP1 (Anti-RPLP1 antibody (Abcam 121190); dilution (1:300). IHC was performed following the recommendations of the manufacturer using VectaStain ABC system (Vector laboratories, Inc.). Protein localization was identified as a positive reaction in dark brown coloring on the tissue slides.

For H-Score assessment, the level of protein was quantitated in each cell type (epithelial, stroma, etc.) indicated as regions of interest (ROI). We selected several sections as ROI within each endometrial biopsy and, due to the limited amount of tissue in endometriotic lesions compared to eutopic endometrial biopsies, we quantitated the entire lesion cross- section as the ROI. The reaction product/signal intensity was then scored as 0 (absent), 1 (weak), 2 (moderate) or 3 (strong) and the percent of cells expressing these levels of intensity were calculated in each ROI. The H-SCORE was then obtained from this information (average level of intensity X percent of cells at that intensity) for semi-quantitative measurement of the positive staining.

Statistical analysis

To test the null hypothesis that the mean levels of RPLP1, miR-451a, and CCNE1 levels in ectopic endometriosis and eutopic endometriosis patients are the same, versus the alternative that the mean level in ectopic endometriosis patients is higher, a paired one-tailed t-test (0.05 significance level)

was used. For correlation studies of RPLP1 V1, V2, CCNE1, and miR-451a delta ct values in each lesion, Pearson's correlation was used. For comparison studies of the in vitro 12Z cells treatment groups, one-way ANOVA was used followed by post-analysis using Bonferroni testing or SNK procedure.

Results:

1. MiR-451a regulates the expression of RPLP1 in 12Z immortalized endometriotic epithelium cell line.

We previously reported that the net level of miR-451a expression was increased in endometriotic lesion tissue, but overall, lesion expression is heterogeneous. We also found that there was a correlation between miR-451a expression and cellular proliferation [130].

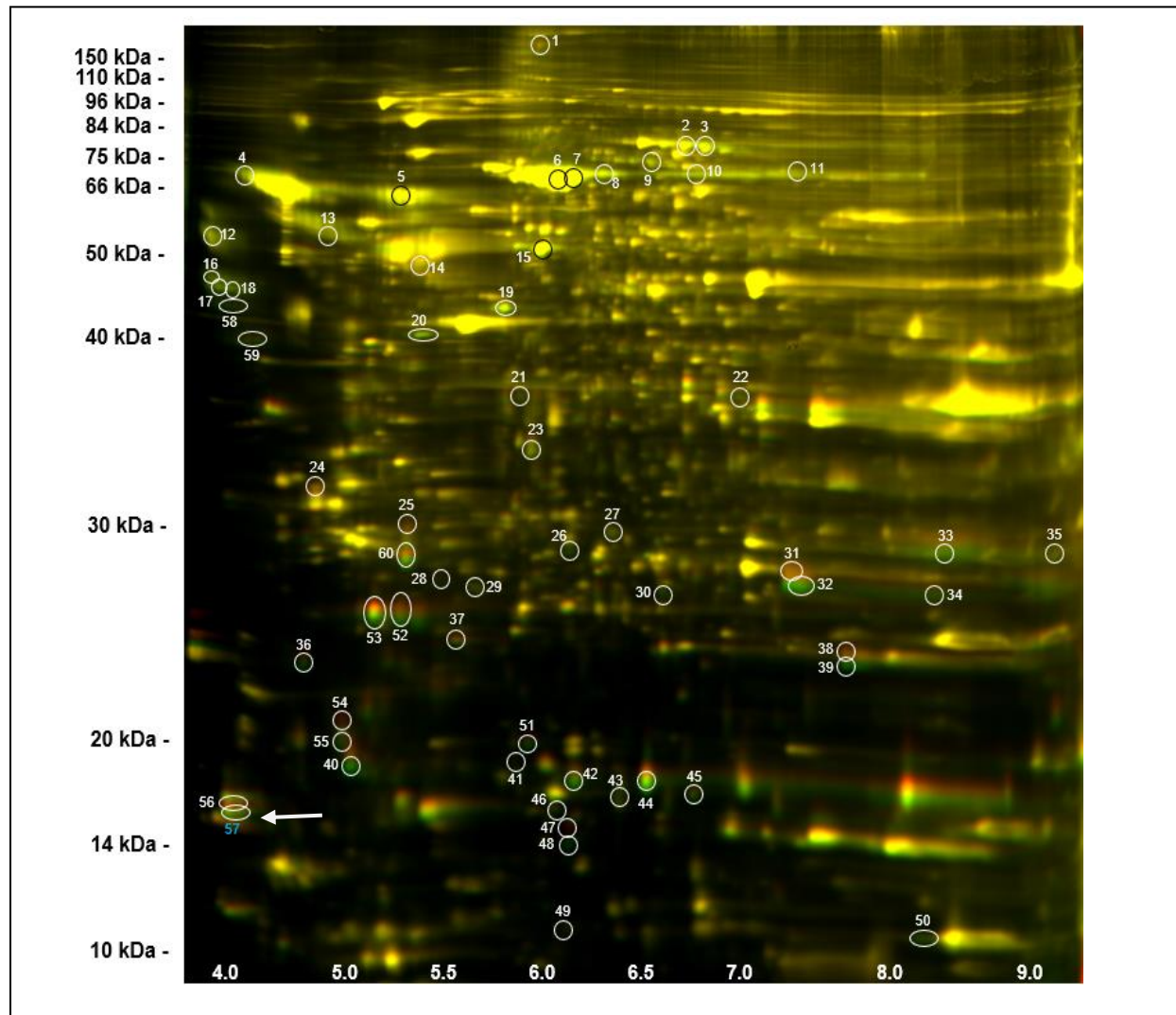
Our recently published data showed that miR-451a level is mis-expressed in human endometriotic lesion tissue, and its level is negatively correlated with cellular survival [159]. To examine the role of miR-451a in human endometriotic lesions, we used our cell model, 12Z immortalized endometriotic epithelial cell line. The cells were transfected with pre-mir-451a, or pre-NT-miR as a (non-targeting) control. Total RNA and protein were extracted at 24h and 48h post-transfection. We focused on 24-hour time point to confirm the detection of early protein changes in response to miR-451a transfection. The isolated proteins were used to run 2-dimensional differential in-gel electrophoresis (2D-DIGE; performed by Applied Biomics, Hayward, CA) experiments to assess the differential protein profiles between transfection groups (Figure 5; this data is representative of 3 separate analysis of different cell transfection studies). Out of hundreds of expressed proteins; 60 proteins were shown to be differentially expressed between targeting and non-targeting cells. We selected twenty-five of these proteins for identification and further analysis for which the ratio changes of the protein differential expression (obtained from in-gel DeCyder software analysis) between pre-miR-451a and pre-NT transfected cells in which protein expression was >1.5-fold. Proteins were then isolated followed by identification using a 5800-mass spectrometer (AB Sciex). We selected protein

(number 61) to be our protein of interest and was identified as RPLP1, Ribosomal protein Large P1.

Figure 5. Proteomic analysis of endometriotic epithelial cells (12Z) by CyDye switch and 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE). 12Z cells were transfected with pre-miR-451a or pre-NT, protein was isolated and then labelled with Cy5 (red) and Cy3 (green), respectively. Over-expressed proteins in pre-miR-451a transfected cells are shown in red, and over-expressed proteins in pre-NT transfected cells are shown in green. The most differentially expressed proteins were marked (circle and number). Out of 60 proteins marked in the gel, we identify a few proteins including protein number #57, which was identified as RPLP1 (arrow).

Vertical axis (Molecular weight), horizontal axis (isoelectric point).

Figure 5



2. MiR-451a inhibits RPLP1 transcript and protein expression:

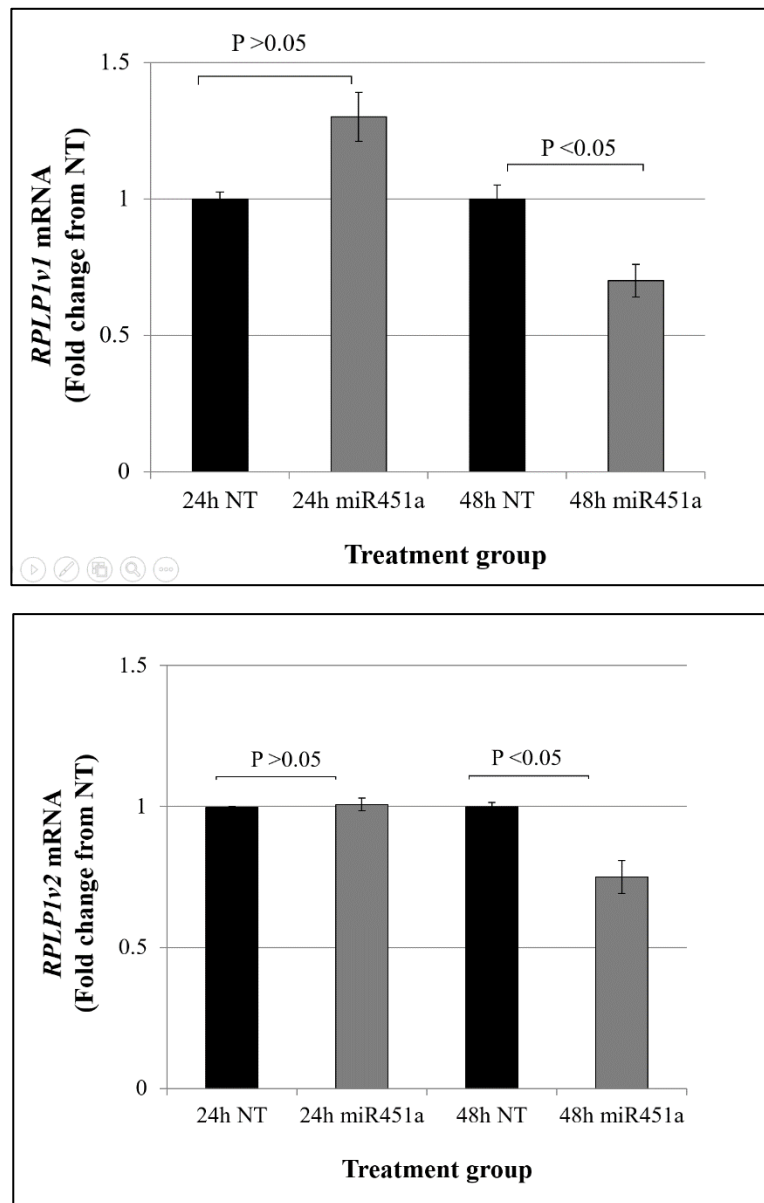
To confirm our initial finding in 2D gel analysis that RPLP1 is down-regulated by miR-451a, we examined RPLP1 mRNA and protein expression in 12Z cells transfected with either pre-miR-451a or pre-NT (negative control). Protein and RNA were isolated at 24h and 48h post-transfection and qRT-PCR and Western analysis were performed (n=3). As displayed in Figure 6A, RPLP1 mRNA level was significantly reduced in pre-miR-451a transfected cells compared to the negative control at 48 h post-transfection ($P<0.05$; $N=3$). In contrast, insignificant increase in the expression of RPLP1 was seen at 24h ($P>0.05$; $N=3$). To confirm our findings on the protein level, we examined the protein expression in the same set of cells by Western blot analysis. As depicted in Figure 6B, RPLP1 protein demonstrated a reduction at 48h post-transfection in 12Z cell transfected with pre-miR-451a compared to pre-NT transfected cells (n=3).

Figure 6. miR-451a reduces the expression of RPLP1 mRNA and protein. Immortalized human endometriotic epithelial cell line (12Z) were transfected with miR-451a or pre-NT (negative control)

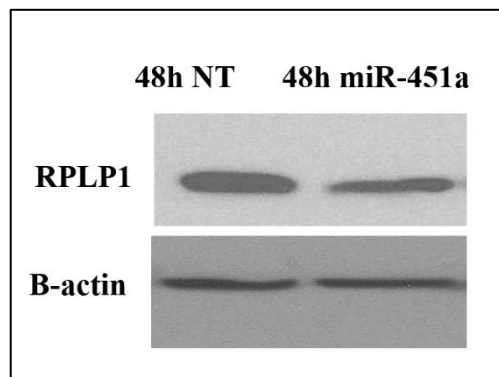
- (A) qRT-PCR analysis of RPLP1 expression in 12Z cells after transfection with pre-miR-451a or pre-NT. At 24 h post-transfection, RPLP1 transcript was insignificantly higher in 12Z cells transfected with pre-miR-451a compared to negative control ($P>0.05$; $N=3$). In contrast, the level of RPLP1 mRNA was significantly reduced 48 h post-transfection in miR-451a transfected cells compared to the pre-NT transfected cells ($P<0.05$; $N=3$). Data were normalized to the level of 18S transcript, and represented as the fold change from control (pre-NT) \pm SEM. Data were analyzed by one-way ANOVA followed by Bonferroni post-hoc analysis.
- (B) Western blot analysis of RPLP1 protein in 12Z cells transfected with either miR-451a or pre-NT (negative control). At 48h post-transfection, RPLP1 protein decreases in miR-451a transfected cells.

Figure 6

A



B



3. RPLP1 expression is increased in endometriotic lesions and it localizes to glandular epithelium:

Based on the above *in vitro* observation that miR-451a may inhibit the expression of RPLP1 in our cell model, 12Z cell line, we hypothesized that miR-451a reduces lesion survival and growth in a pathway that involves RPLP1.

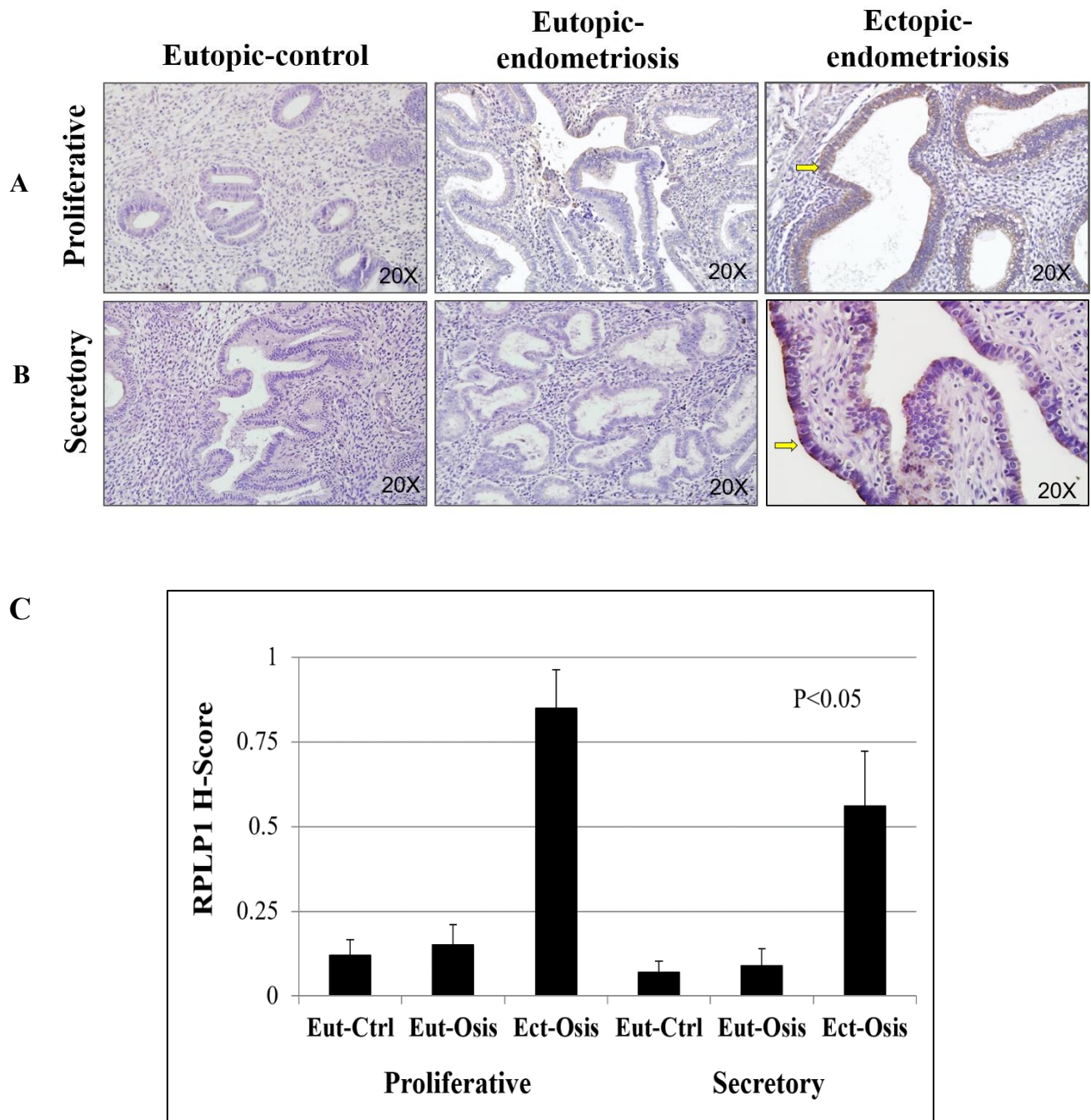
To test this hypothesis, we first wanted to confirm and localize the expression of RPLP1 in human endometriotic lesion tissue. To do so, we conducted a preliminary assessment using a total of 60 archived eutopic (N=10 secretory stage, N=10 proliferative stage) and matched ectopic (endometriosis) endometrial tissues from women with confirmed endometriosis (N=10 secretory stage, N=10 proliferative stage) as well as eutopic endometrium from women without endometriosis as a control (N=10 secretory stage, N=10 proliferative stage). Samples were obtained from the University of Kansas Medical Center, Department of Pathology and Laboratory Medicine, and classified as either proliferative or secretory stage of the menstrual cycle. For this preliminary study, all women with endometriosis had stage I/II disease. Samples were prepared for immuno-histochemical localization of RPLP1 as described in (“Materials and Methods”) section. As depicted in Figure 7A-B, RPLP1 was localized primarily to the glandular epithelium (indicated by the arrows) and its expression (indicated by intense brown staining) was substantially elevated in the ectopic lesions of endometriosis women compared to matched eutopic tissues, and eutopic endometrium from women free of endometriosis. More importantly, RPLP1 expression in ectopic lesion from women in proliferative stage of the menstrual cycle (Figure 7A) was significantly higher compared to ectopic lesion from women in the secretory stage of the menstrual cycle (Figure 7B). To further assess the expression of RPLP1 in these tissues, we used the H-Score system which allows for a semi- quantitative measurement of the immunohistochemical

positive staining. As displayed in figure 7C, the H-score of the ectopic tissues was higher than the matched eutopic and the eutopic control, and this elevation is more predominant in the proliferative phase of the menstrual cycle. All in all, these results agree with the observation that ectopic lesion tissues from the proliferative stage of the menstrual cycle are highly proliferative and this observation supports our overall hypothesis.

Figure 7. RPLP1 expression is elevated in human ectopic lesion tissue and localized in the glandular epithelium. Immunostaining for RPLP1 in ectopic endometriotic tissue and matched eutopic tissues from women with confirmed endometriosis, and eutopic control from women without endometriosis **(A)** in the proliferative stage of menstrual cycle **(B)** in the secretory stage of menstrual cycle. The arrow illustrates the location of RPLP1 protein in glandular epithelium of the ectopic tissues (brown color indicates positive staining and was scored as 3 = strong, 2 = moderate, 1 = weak and 0 = absent). The intensity of the staining, which indicates the degree of expression, is elevated in the ectopic tissue from patients with endometriosis compared to matched eutopic and eutopic control, which show very light expression of RPLP1. All magnifications were X20

(C) The immunohistochemical histological score (H-score) of RPLP1 in endometrial tissues (ectopic and eutopic) in both stages of menstrual cycle. H-score significantly increases in ectopic tissue from patient with endometriosis, and it is more elevated in proliferative stage compared to the secretory stage. (controls; N=30, P< .05). Data are presented as the mean \pm SEM and were analyzed by one-way ANOVA followed by Bonferroni post-hoc analysis.

Figure 7



4. RPLP1 mRNA and protein is highly expressed in human ectopic endometriotic lesions and correlates with lesion proliferation

To confirm the above finding that RPLP1 is highly expressed in human ectopic lesions, we utilized human tissues (eutopic endometrial tissue from women with endometriosis, N=55, and matched ectopic lesions, N=77) from the Cleveland Clinic, Department of Obstetrics and Gynecology. We first analyzed RPLP1 (variant 1 and variant 2) mRNA expression, by qRT-PCR, in matched eutopic and ectopic tissue from women with endometriosis. Stage of endometriosis (I/II versus III/IV) did not influence expression of RPLP1 so data were collapsed and analyzed as eutopic versus ectopic endometrial tissue. As depicted in figure 8A, the fold change of expression (ratio of lesion/eutopic) of RPLP1 V1 was greater (>1.0) in 67% (52/77) of ectopic endometriosis lesion tissue compared to matched eutopic endometrium, however, 32% (25/77) of the lesions expressed lower level of RPLP1 V1 (≤ 1.0). Similar to this, the fold change of expression of RPLP1 V2 (figure 8B) was greater (>1.0) in 54% (42/77) of ectopic endometriosis lesion tissue compared to matched eutopic endometrium and lower (≤ 1.0) in 45% (35/77) of the lesions.

The overall mean fold change of expression of both variants was significantly higher (3.72-fold increase; $P<0.01$) in ectopic lesion compared to eutopic tissues as shown in figure 8C. Since we have two variants of RPLP1, we correlated their expressions in the same lesions, which showed positive correlation as depicted in figure 8D (Pearson $r = 0.8542$; $P<0.0001$). We next performed Western blot analysis to examine the expression of RPLP1 at the protein level in matched eutopic and ectopic tissue from women with endometriosis. As displayed in figure 8E, RPLP1 protein was greater in ectopic lesion tissues compared to matched eutopic endometrium. Changes in protein expression were in agreement with changes in mRNA expression. To compare the expression of

miR-451a and RPLP1, we measured the total expression level of miR-451a in these lesions. As shown in figure 9A 57% (34/59) of the ectopic lesions expressed higher level (>1.0) of miR-451a, and 43% (25/59) express lower level (≤ 1.0) compared with matched eutopic tissue. Furthermore, the correlation study performed demonstrated that miR-451a is negatively associated with RPLP1 V1 (Pearson $r = -0.32$; $P < 0.001$; $N = 73$) in the same lesion as displayed in figure 9B. This observation suggested to us that high levels of miR-451a were associated with lower levels of RPLP1 and might suggest an anti-proliferative role of miR-451a via modulation of RPLP1 expression.

To test the hypothesis that RPLP1 plays a role in inducing the survival and proliferation of endometriotic lesion tissue, we evaluated the expression of endometriotic lesion markers which we have previously demonstrated as indicators of lesion “survival status”; we assessed the cell proliferation marker (CCNE1) in the same tissues samples that were analyzed above. Figure 10A shows insignificant elevation of CCNE1 mRNA in ectopic lesion tissues compared to matched eutopic tissues (1.65-fold increase; $P > 0.05$), consistent with the elevation of RPLP1 mRNA. This observation was confirmed by the correlative study illustrated in Figure 10B that demonstrated that RPLP1 expression is positively correlated with the proliferative marker CCNE1 (Pearson $r = 0.571$; $P < 0.01$; $N = 69$).

To this point, we interpret these data to suggest that the up-regulation of RPLP1 in endometriotic lesions and its correlation with CCNE1 may indicate a potential function in lesion survival and growth.

Figure 8. RPLP1 mRNA and protein are elevated in endometriotic lesions. Endometriotic lesions and matched eutopic tissue were utilized for RNA and protein isolations for RPLP1 and CCNE1.

(A) qRT-PCR analysis of RPLP1 V1 in ectopic lesion shows fold change in expression (from highest to lowest) for RPLP1v1. Note that 25/77 lesions expressed lower levels of RPLP1v1 (≤ 1.0) compared to matched eutopic endometrium and 52/77 lesions expressed higher levels of RPLP1v1 (> 1.0). Please notice that RPLP1 expression ranges from about 10-fold lower in some lesions to approximately 100-fold higher in others compared to matched eutopic tissue.

(Note that sample numbers 74 -77 were capped at a 9-fold increase in this figure; actual fold increases for samples 74, 75, 76 and 77 were 12.56, 33.40, 38.81, 99.27 -fold higher, respectively.)

Figure 8

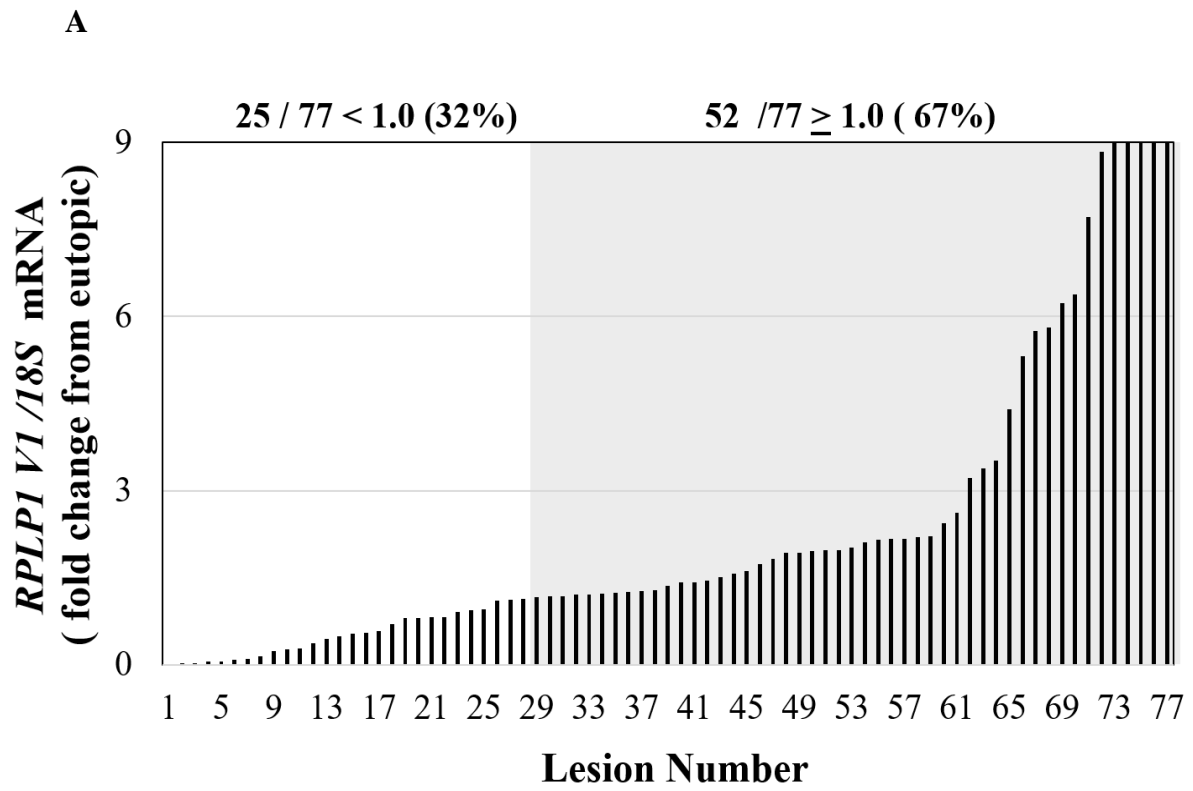


Figure 8 (continued). RPLP1 mRNA and protein are elevated in endometriotic lesions. Endometriotic lesions and matched eutopic tissue were utilized for RNA and protein isolations for RPLP1 and CCNE1.

(B) qRT-PCT analysis of RPLP1 V2 in ectopic lesion shows fold change in expression (from highest to lowest). Note that 35/77 lesions expressed lower levels of RPLP1v2 (≤ 1.0) compared to matched eutopic endometrium and 42/77 lesions expressed higher levels of RPLP1v1 (> 1.0).

(Note that sample numbers 75 -77 were capped at a 9-fold increase in this figure; actual fold increases for samples 75, 76 and 77 were 10.27, 18.86, 24.69 -fold higher, respectively.)

Figure 8

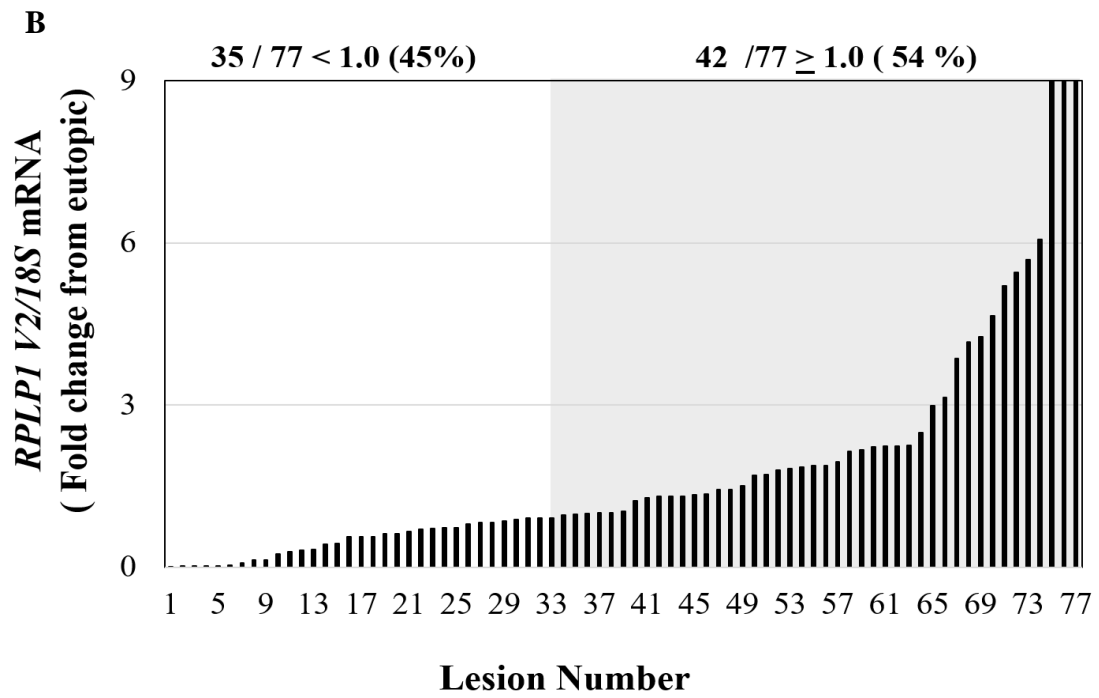
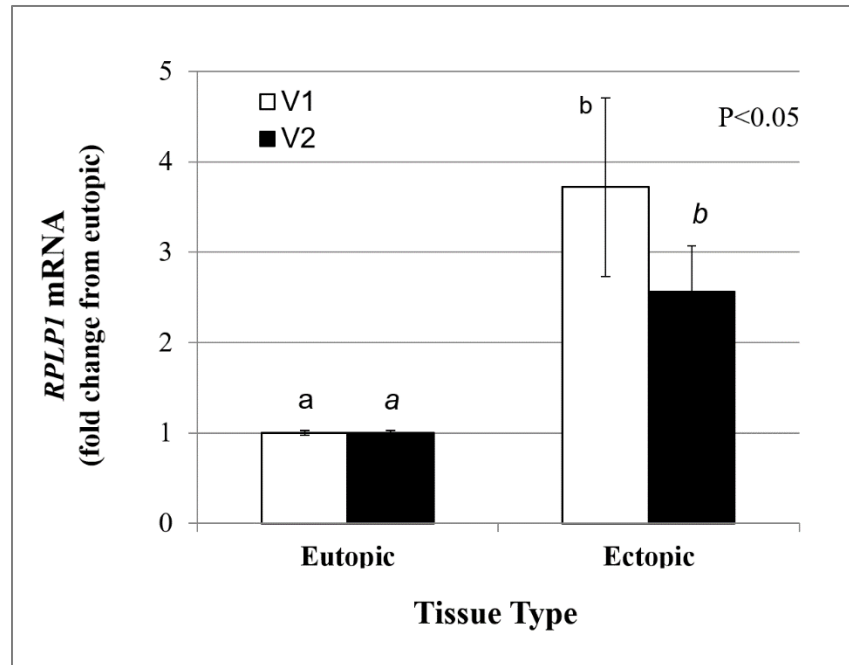


Figure 8 (continued). RPLP1 mRNA and protein are elevated in endometriotic lesions. Endometriotic lesions and matched eutopic tissue were utilized for RNA and protein isolations for RPLP1 and CCNE1.

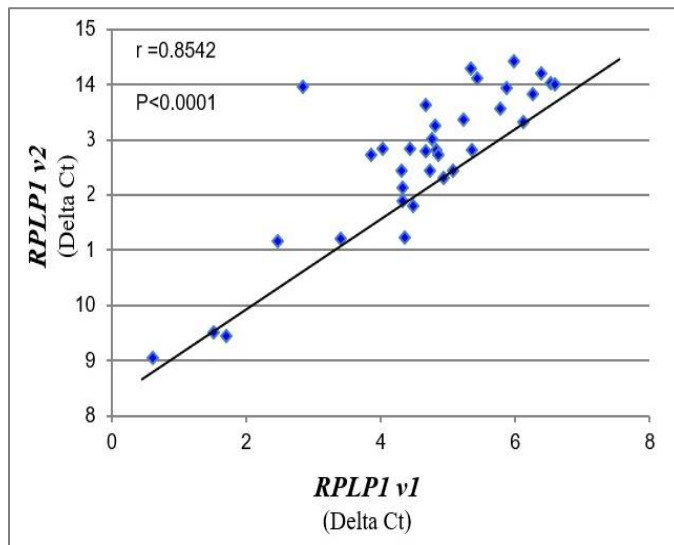
- (C) qRT-PCR analysis of RPLP1 (V1 and V2) expression in endometrial tissues demonstrates significant increase of the mean fold changes of both variants in the ectopic lesions compared to eutopic tissues higher. (3.72-fold increase; $P < 0.01$)
- (D) Comparative analysis of the delta ct values of RPLP1 V1 and V2 in the same sample shows positive correlation of the expression of both variants. (Pearson $r = 0.8542$; $P < 0.0001$)
- (E) Western blot analysis of RPLP1 protein shows elevation in the ectopic lesion compared to eutopic tissues. Values were normalized to B-actin.

Figure 8

C



D



E

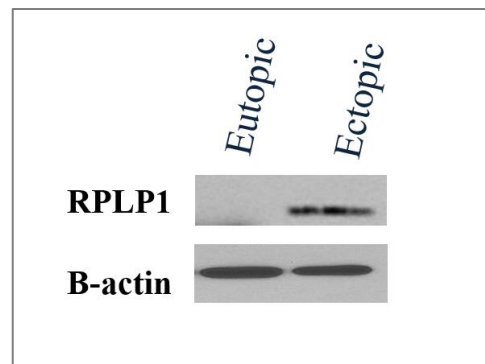
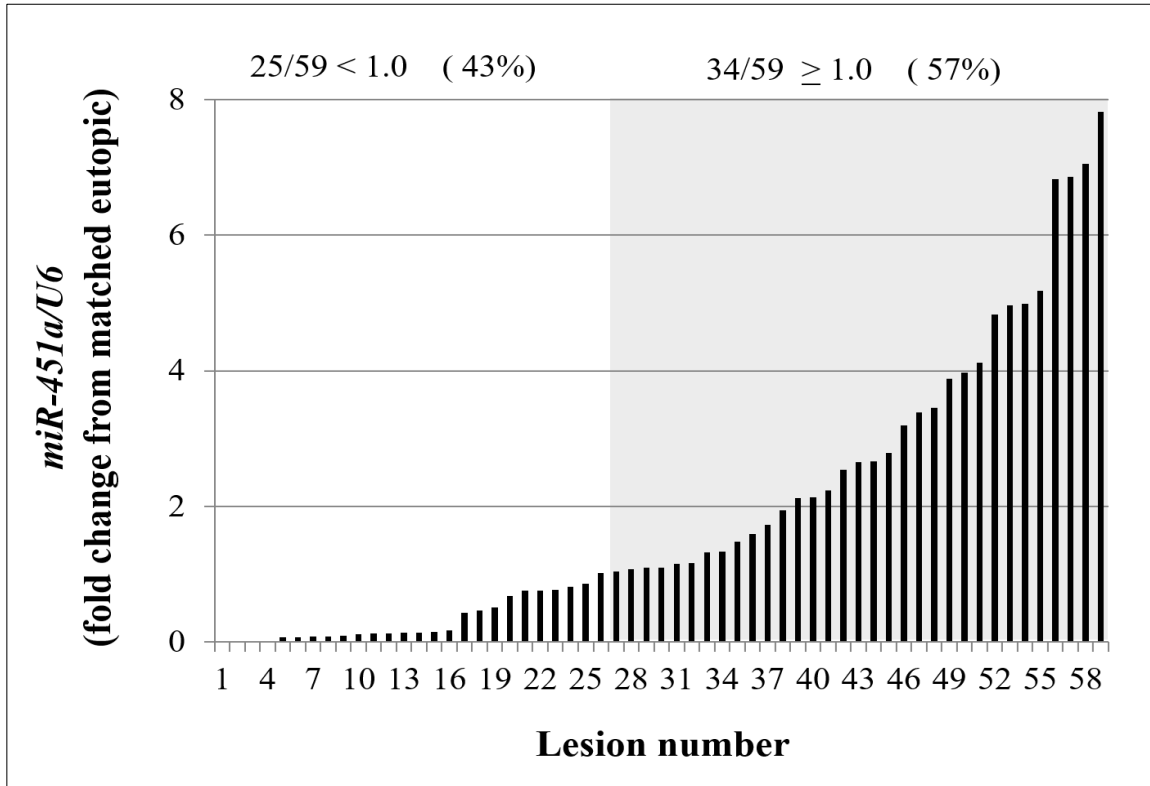


Figure 9. The expression of miR-451a transcript is heterogenous and negatively associated with the expression of RPLP1 V1 in endometriotic lesions. Endometriotic lesions and matched eutopic tissues were processed for RNA isolation and for the examination miR-451a transcript level.

- (A) qRT-PCR analysis of miR-451a which demonstrates the fold change of expression of miR-451a is differentially expressed among all lesions. Note that 34/59 shows higher level (>1.0) of miR-451a transcript compared to matched eutopic, and 25/59 shows a reduction in ectopic lesions (≤ 1.0).
- (B) Represented Delta ct values for miR-451a and RPLP1 V1 in the same sample. Lower delta ct values correspond to a higher level of mRNA expression. Endometriotic lesions which express high levels of miR-451a (lower left corner of graph) expressed low levels of RPLP1 V1, and the lesions which express lower levels of miR-451a mRNA (upper right corner of graph) express higher levels of RPLP1 V1 mRNA. A Comprehensive analysis of 73 total lesions demonstrated a negative correlation between miR-451a and both variants of RPLP1. (Pearson $r = -0.32$; $P < 0.001$; $N = 73$).

Figure 9

A



B

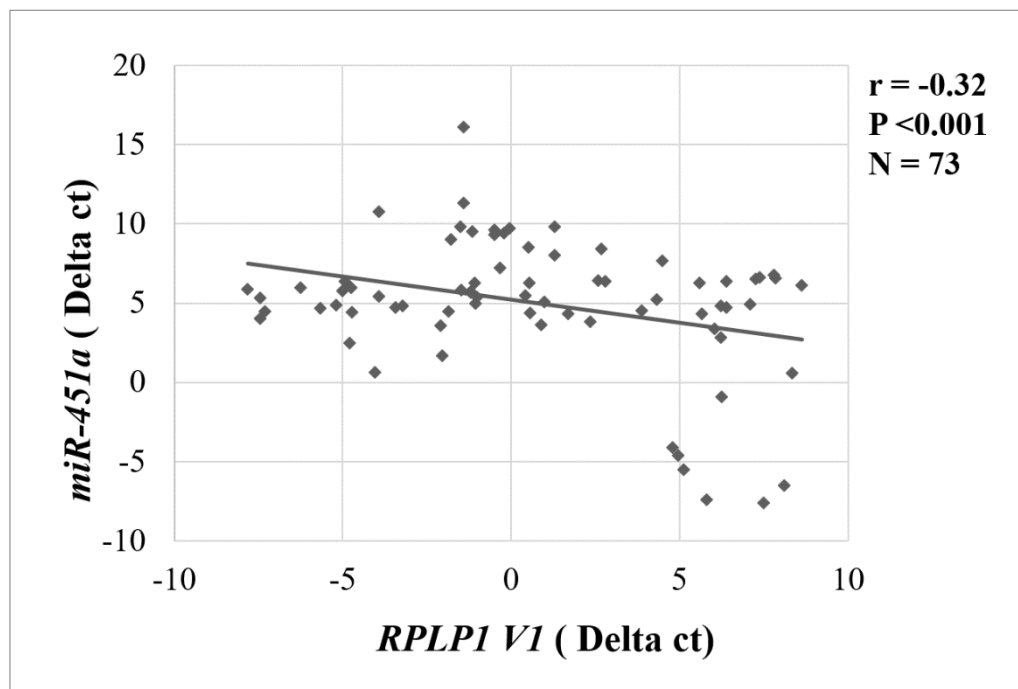
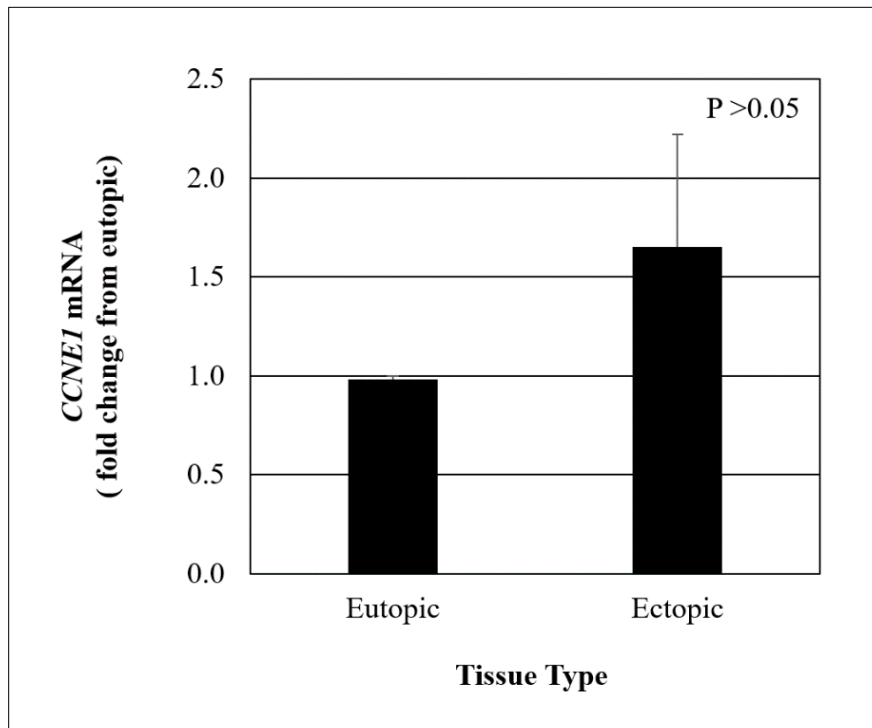


Figure 10. RPLP1 mRNA and protein are positively correlated with the expression with the proliferation marker, CCNE1.

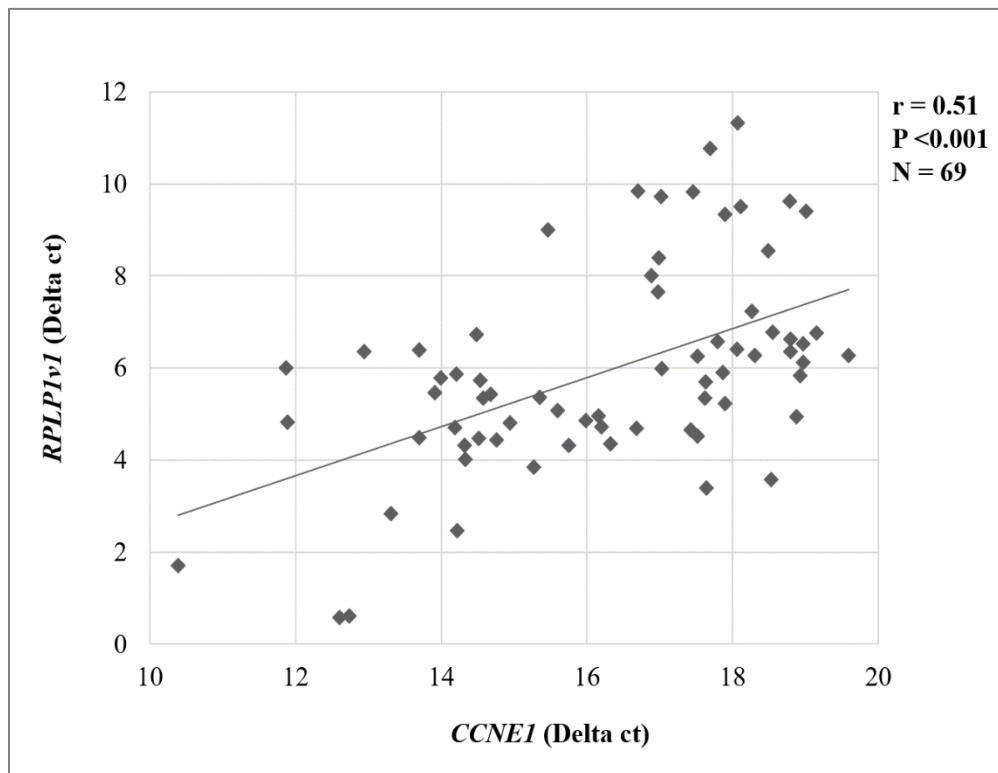
- (A) qRT-PCR analysis of CCNE1 expression in endometrial tissues illustrates a non-significant increase (1.65-fold increase; $P>0.05$) of the mean fold change in the ectopic lesions compared eutopic tissues.
- (B) Delta ct values for RPLP1v1 and CCNE1 in the same sample. Lower delta ct values correspond to a higher level of mRNA expression. Endometriotic lesions which express high levels of RPLP1 (lower left corner of graph) also expressed high levels of CCNE1, while lesions which express lower levels of RPLP1v1 mRNA (upper right corner of graph) express lower levels of CCNE1 mRNA. Overall assessment of 69 total lesions revealed a positive correlation between transcripts. (Pearson $r = 0.571$; $P<0.01$; $N=69$). RPLP1, CCNE1 data were normalized to the level of 18S transcript, and represented as the fold change from control (eutopic tissues) \pm SEM.

Figure 10

A



B



5. RPLP1 enhances cellular proliferation and survival in 12Z immortalized endometriotic epithelial cells

Our data confirmed the up-regulation of RPLP1 transcript and protein levels in human endometriotic lesions, which might be controlled by miR-451a. Furthermore, we established that the expression of RPLP1 in human endometriotic lesions is positively correlated with the expression of CCNE1, a proliferative marker. RPLP1 was already identified as survival and proliferation promoter in tumors, accordingly, we wanted to determine if RPLP1 plays similar role in endometriosis by enhancing the growth and establishment of the endometriotic lesions. To test this hypothesis, 12Z cells were transfected with non-targeting (negative control) or siRNA to RPLP1 (n=3), which provided transient knockdown of RPLP1. Following this, RNA was collected at 24, 48, and 72 h post-transfection to assess the expression of cell proliferation marker cyclin E1 (CCNE1), and tumor suppressor marker phosphatase and tensin homolog (PTEN). RPLP1 knockdown was associated with a reduction in the proliferation marker (CCNE1) (Fig. 11 A) and an elevation of tumor suppressor marker (PTEN) (Fig. 11 B). To confirm RPLP1 knockdown, qRT-PCR ($P<0.05$; N=3) (Figure. 11 C) and protein analysis (Figure. 11D) were performed for RPLP1. This alteration in markers expression was not noticed in control 12Z cells transfected with non-targeting siRNA

Since we found that 12Z cells express high levels of RPLP1, the transient transfection with siRNA, may not be optimal for long-term studies. This postulation was supported by data displayed in figure 11C which shows that 12Z cells transfected with siRNA for RPLP1 re-expressed RPLP1 transcript in 72 hours post-transfection.

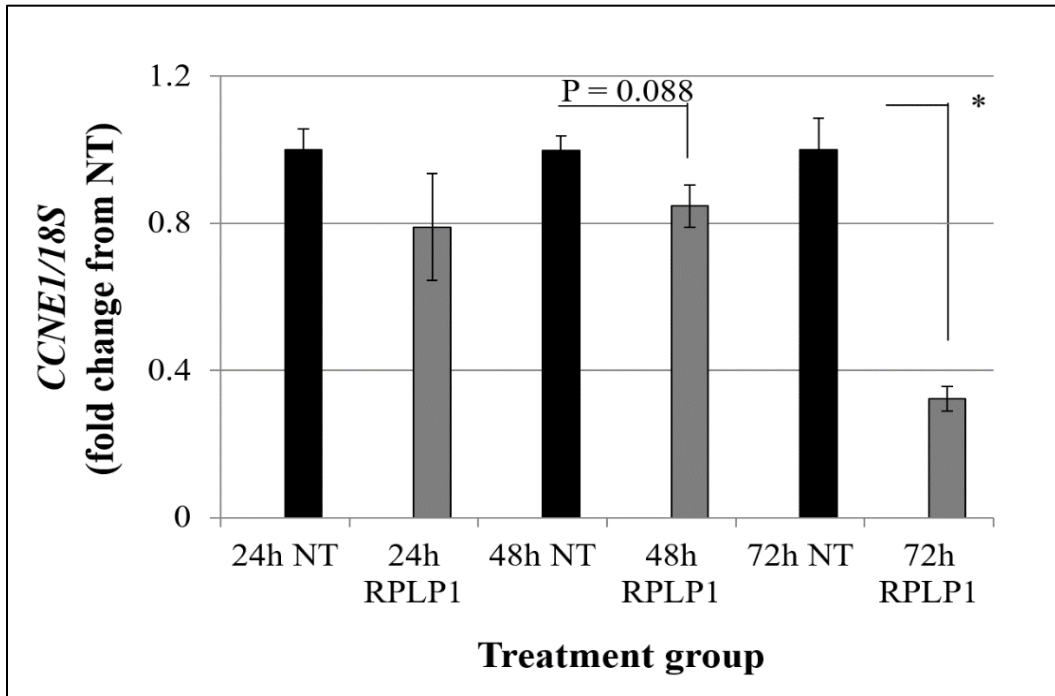
To address this concern, we developed stable RPLP1 deficient cell lines to be utilized in further experiments. To achieve this goal, 12Z cells were transduced with GFP- lentiviral pseudoparticles

containing a non-targeting cDNA or shRNA to RPLP1 (N=3) (Genecopoeia, Rockville, MD). To confirm the transduction, cells were cultured with puromycin (2 µg/ml) for 72h to select positively infected cells. As displayed in Figure 12 A-B, endogenous depletion of RPLP1 resulted in cell death and a significant decrease in cell survival in the lenti-virus infected cells. collectively, these data support a potential role of RPLP1 in cellular proliferation/survival in endometriotic epithelial 12Z cells.

Figure 11. RPLP1 Knockdown reduced cell survival and pro-apoptotic markers. Immortalized human endometriotic epithelial cell line (12Z) were transfected with siRNA for RPLP1, or non-targeting siRNA (negative control), and represented as the fold change from control (non-targeting) \pm SEM. RNA and protein were isolated from the cells at 24h, 48h, and 72h post-transfection to perform qRT-PCR analysis for CCNE1 (A), PTEN (B), and RPLP1 V1 ($P < 0.05$; $N = 3$) (C). Protein lysates were used for western blot for RPLP1 detection (D)

Figure 11

A



B

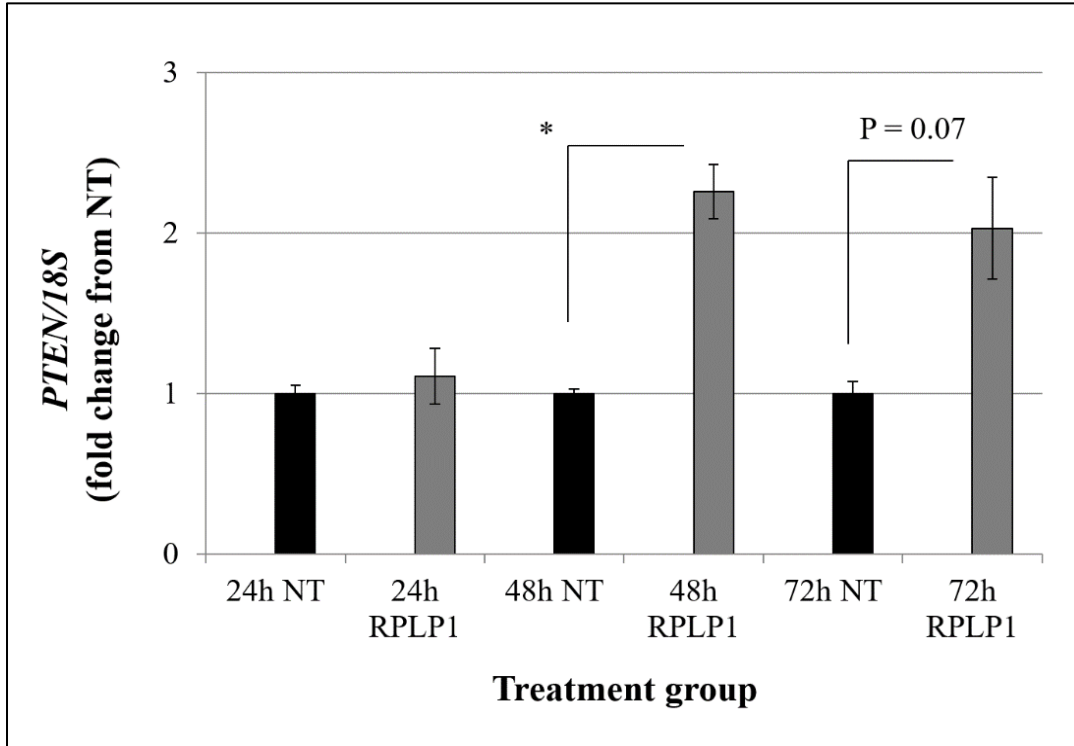
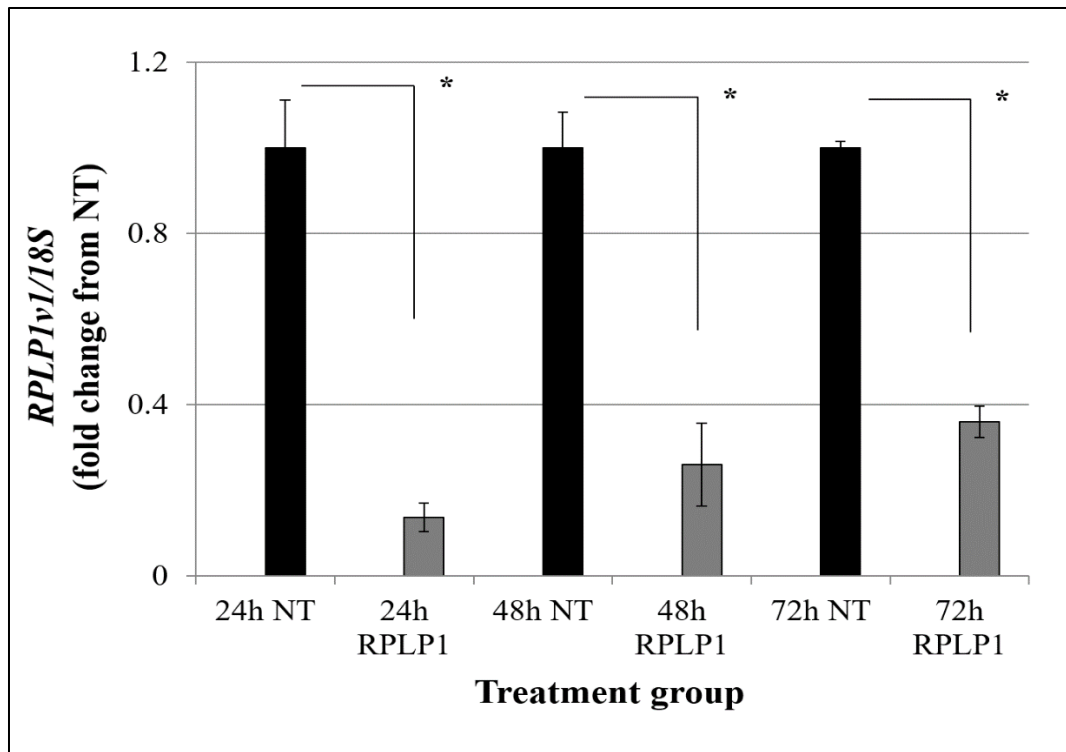


Figure 11

C



D

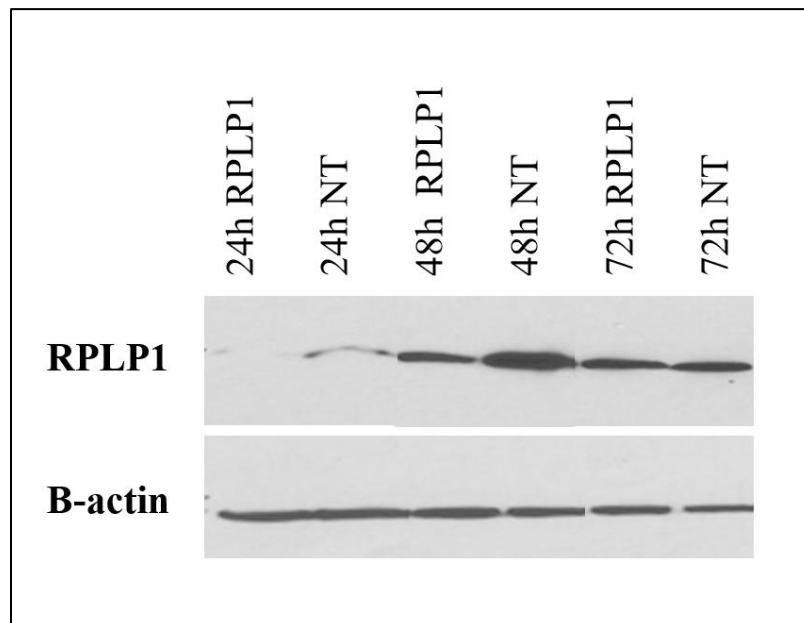
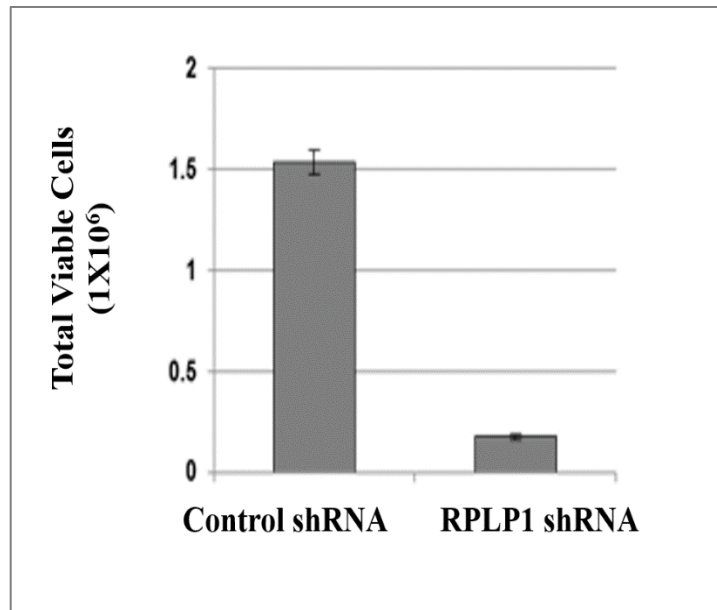


Figure 12. Establishment of stable 12Z cell line with RPLP1 knockdown by lentiviral pseudoparticles resulted in reduction in cell survival. Endometriotic epithelial cells (12Z) was transfected with GFP- lentiviral pseudoparticles containing a non-targeting cDNA or shRNA for RPLP1, the cells were cultured and treated with puromycin after 48h for positive cells selection. The cells then were collected 72 h post transfection (n=3).

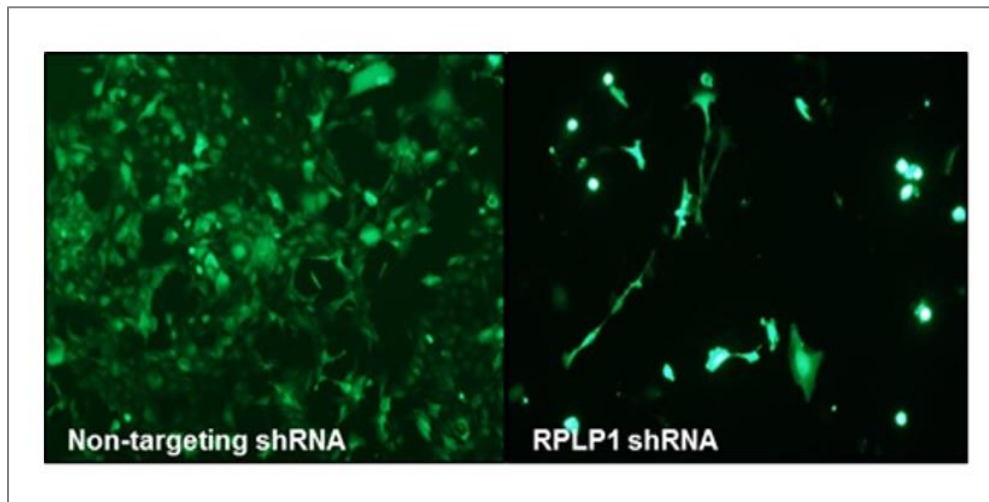
- (A) The viability of 12Z cells was determined by the exclusion of Trypan Blue. The number of viable 12Z cells transfected with ShRNA for RPLP1 was significantly reduced compared to the 12Z control ($P < 0.001$).
- (B) Fluorescent microscopy of the 12Z cells demonstrated a decrease in the number of cells transfected with ShRNA for RPLP1 (green fluorescence) compared to the 12Z cells transfected with non-targeting cDNA.

Figure 12

A



B



Discussion:

Endometriosis is a disease characterized by the establishment, growth and survival of ectopic endometrial tissue (endometriotic lesion). miR-451a is already established as an oncogene in several tumors including breast, liver, and bladder cancer by suppressing the expression of genes responsible of cell growth, survival and angiogenesis [190-194] . Thus, it is not of a surprise that a similar role of miR-451a was also found in endometriosis. Endometriotic lesions share similar characteristics to tumors such as higher proliferative and growth rate, increase inflammation, estrogen responsiveness, and gene mutations [195-199].

It should be noted that the expression of miR451a in endometriotic lesions is heterogeneous and was correlated with cellular proliferation [131, 159]. This finding was confirmed by a previous study in our laboratory that showed that the mean fold change of miR-451 (now referred to as miR-451a) expression was significantly higher in ectopic lesions compared to eutopic tissues [159]. The elevation of miR-451a was negatively associated with the expression of CCNE1, a proliferation marker, and positively with PTEN, a proapoptotic marker [159, 200], which supports its role as tumor suppressor in endometriotic lesion tissue. The reason for the heterogeneous expression may be due to the “proliferation status” of the lesion where newly formed lesions express low levels of miR-451a and high levels of CCNE1 and are thus more proliferative. We postulate that as the lesions progress and eventually regress and become necrotic, miR-451a levels rise while CCNE1 levels decrease and the lesion eventually regresses. This postulate is currently being explored in our laboratory.

In this study, we wanted to gain additional insight into the mechanism by which miR-451a reduces cellular proliferation in endometriosis. Forced expression of miR-451a in 12Z endometriotic

epithelial cells showed downregulation of ribosomal protein, RPLP1, in 2-DiGE analysis which was confirmed in a separate series of 12Z cell transfections both at the mRNA and protein level. RPLP1 is a ribosomal protein that forms the ribosomal stalk in 60S subunit, and aids in translation [201]. In gynecological tumors, RPLP1 was found to be highly expressed and was correlated with cellular proliferation [179]. Overall, there are limited studies exploring the physiological role RPLP1 in disease development, however, those data that are available support the notion that RPLP1 is essential for cellular growth and proliferation. Based upon this, we hypothesized that RPLP1 expression is related to the establishment and survival of endometriotic ectopic lesions. This study is the first to identify the expression of RPLP1 in endometriotic lesion tissue and its potential regulation by miR-451a. Our studies define yet another novel pathway which may contribute to the pathophysiology of endometriosis. Future studies will explore the targeting of this pathway as a potential, non-hormonal therapy for endometriosis.

To begin to explore the function of RPLP1, we first evaluated its level of expression in human samples. Immunohistochemistry staining showed higher expression of RPLP1 in the epithelial tissues taken from patients with endometriosis versus disease-free women, and this elevation was higher in the proliferative stage of menstrual cycle compared to secretory stage. As the proliferative stage is when the endometrium grows and thickens, the higher level of RPLP1 in the diseased tissues may accelerate the process. In further analysis, RPLP1 RNA and protein in the ectopic lesions were higher compared to matched eutopic tissue from the same patient. RPLP1 RNA expression was higher (>1) in 67% (for RPLP1 V1) and 54% (for RPLP1 V2) in ectopic lesions versus paired eutopic tissues. Further data that support the the notion that RPLP1 may be associated with cellular proliferation of endometrial tissue/cells is that the expression of RPLP1 in

these lesions demonstrated a positive correlation with the expression of Cyclin E1 (CCNE1), a proliferative marker.

Our data suggests that RPLP1 expression could be suppressed by miR-451a in endometriosis which causes increase in lesion proliferation. Thus, we used the same lesions examined for RPLP1 to investigate the expression of miR-451a. We assumed that miR-451a will be lower in the ectopic lesions and this will explain the up-regulation of RPLP1. Contradictory to our assumption, miR-451a mRNA showed higher expression in 57% (>1) of the lesions compared to matched eutopic tissues. Thus, we correlated the levels of miR-451a and RPLP1 in each lesion individually and found negative correlation, which confirms our hypothesis. It should be noted that the differential expression of miR-451a and RPLP1 in these samples could be caused by the status of these lesions upon isolation. The heterogeneity of the endometriotic tissues is a hallmark of the disease, which creates difficulty in providing consistent data. Thus, the utilization of in vitro cell model, such as 12Z cells, is highly preferred to overcome this issue in endometriosis research.

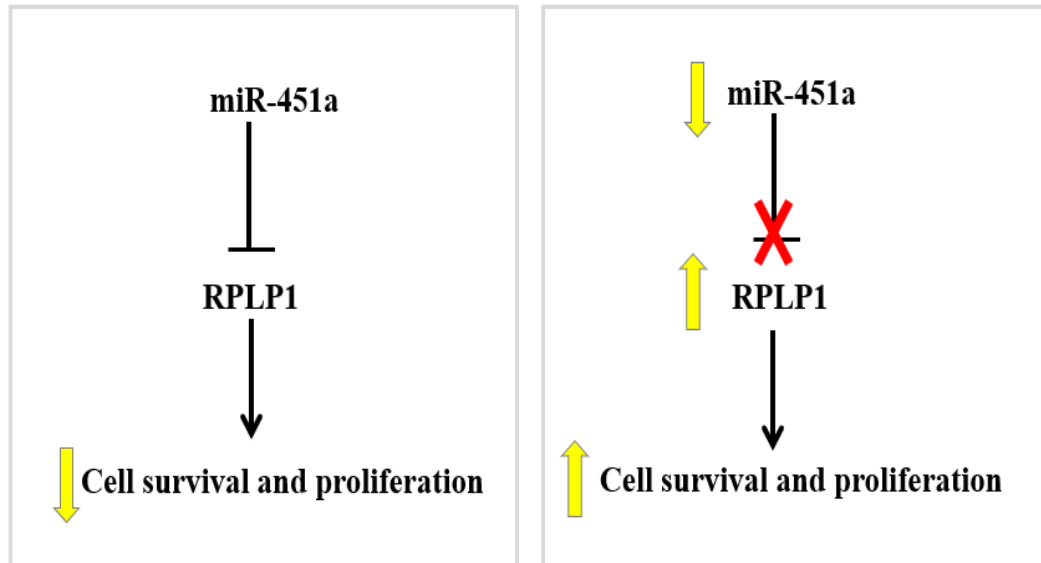
In our cell model, 12Z cell line, the reduction of RPLP1 by siRNA demonstrated an up-regulating of PTEN, pro-apoptotic marker, and downregulating of CCNE1, proliferative marker, which indicated a reduction in proliferation and survival. The logic of using these markers for assessing cellular proliferation is the lack of evidence that either of these proteins are direct targets of miR-451a. However, because of the transient transfection established by siRNA, the level of RPLP1 started to increase at 72h post-transfection. Thus, we used GFP- lentiviral pseudoparticles containing shRNA to RPLP1 which caused a reduction in cell number.

Overall, these data support previous studies that show the tumor suppressing effect of miR-451a in endometriosis [131, 159] and identify a new gene target, RPLP1, that regulates cell

proliferation (Figure 13). In next chapter, we discussed a potential pathway that explains how miR-451a regulates the expression of RPLP1.

Figure 13. The anti-proliferative effect of miR-451a is assumed to be partially through regulating the expression of RPLP1, which plays a role in driving cellular growth.

Figure 13



Chapter III:

**miR-451a-c-Myc pathway regulation of 60S acidic ribosomal protein P1
(RPLP1) expression: A novel pathway in endometriotic lesion tissue survival**

Abstract

Introduction: Endometriosis is a chronic inflammatory disease in women of reproductive age in which endometrial tissue establishes and survives in ectopic locations, yet the mechanisms that drive lesion survival are poorly understood. We have previously identified RPLP1 as a novel factor that modulates endometriotic epithelial cell survival. RPLP1 has been identified as a c-Myc responsive gene in rat fibroblasts (Guo et al., 2000), but a similar pathway has not been established in endometriotic lesion tissue/cells. The objective of the current study was to examine the role of c-Myc in regulating RPLP1 expression in endometriotic lesion tissue and epithelial cells to decipher the role of this protein in endometriosis pathophysiology.

METHODS: c-Myc and RPLP1 mRNA was assessed using qRT-PCR in paired endometriotic lesion tissue and eutopic endometrium from women with endometriosis (N=77 lesions and N=55 eutopic). RPLP1 regulation by c-Myc and cell survival were assessed in vitro using the endometriotic epithelial cell line, 12Z.

RESULTS: Average expression level of lesion c-Myc mRNA was significantly greater compared to paired eutopic endometrium (4.1-fold increase; $P<0.001$). Lesion c-Myc mRNA levels were positively and significantly correlated with those of RPLP1 (Pearson $r = 0.56$; $P<0.01$). To evaluate if c-Myc modulated 12Z cell RPLP1 expression, 12Z cells were transfected with c-Myc siRNA and RPLP1 mRNA and protein expression were examined. Forced expression of c-Myc siRNA significantly suppressed c-Myc expression as well as that of RPLP1 ($P<0.05$; $N=4$). As miR-451a putatively regulates both c-Myc and RPLP1, we examined if it could modulate their expression. Compared to non-targeting miRNA-transfected 12Z cells, forced expression of miR-451a

significantly reduced both c-Myc mRNA and protein expression as well as that of RPLP1 ($P<0.01$; $N=4$) which was associated with reduced cell survival.

CONCLUSIONS: The average level of expression for both RPLP1 and c-Myc are significantly elevated in endometriotic lesion tissue and display a positive correlation. Further, c-Myc regulation of RPLP1 expression involves post-transcriptional regulation by miR-451a. Our observations continue to support our notion that miR-451a expression modulates endometriotic lesion survival and this regulatory pathway includes RPLP1 and c-Myc.

Introduction:

MicroRNAs are small, non-coding, nucleotides sequences that control gene expression post-transcriptionally [132]. The expression of miRNAs is essential for normal tissues and cellular development and may contribute to many pathological conditions such as cancer, auto-immune and inflammatory diseases [202-205]. The role of miRNAs in developing and maintaining ectopic lesions in endometriosis was investigated immensely [131, 155, 156, 159, 206].

Endometriosis is a chronic inflammatory disease that affects women of reproductive age and contributes to a high morbidity rate [20, 29, 207]. It is characterized by the establishment of endometrial tissues in ectopic locations. Several miRNAs were found to be mis-expressed in women with endometriosis including miR-451a, which is one of the most deregulated miRNAs in endometriotic lesions of patients with endometriosis [131, 152, 154, 159]. Recently, our group demonstrated that the level of miR-451a is negatively associated with endometriotic lesion and epithelial cell survival through potentially targeting RPLP1, Ribosomal Protein Large P1.

Although the essential function of RPLP1 in guiding protein translation is well-established, its role in disease development is still under investigation [208]. The available data suggest that RPLP1 contributes to cellular proliferation, transformation, and tumorigenesis [178, 179, 189]. In addition, Artero-Castro and colleges [179] demonstrated that expression of RPLP1 in gynecological tumors and autoimmune disease is elevated leading to increased cellular proliferation. It was also shown that RPLP proteins are elevated in the endometrial carcinoma that spread to the myometrium tissue [179]. Another study performed to investigate the function of RPLP1 in mammalian development proposed that RPLP1 is important to maintain proper cellular growth and proliferation in neonatal mice. Neocortical RPLP1 knockout caused delays in

proliferation and induced apoptosis of progenitor cells. This was caused by a disturbance of key cell cycle and apoptosis regulators such as cyclin A, cyclin E, p21CIP1, p27KIP1, p53. Upon deletion of RPLP1 in fibroblasts, protein synthesis did not change, but the expression pattern of some proteins was affected, including proteins involved in protein folding and unfolding, apoptosis, and cell signaling [189].

Our finding of RPLP1 elevation in endometriotic lesion tissue and its pro-survival role in endometriosis is the first to identify this novel protein as a player in endometriosis pathophysiology. Furthermore, the expression of RPLP1 was negatively associated with that of miR-451a. Thus, RPLP1 was predicted to be a potential target of miR-451a. In this study, we want to explore this regulation and identify additional mediators that are involved in the miR-451a-RPLP1 pathway. This was achieved by using bioinformatic resources to determine if the miR-451a seed sequence binds to the 3'UTR of RPLP1, the main mechanism that allows miRNA to repress gene expression. In addition, we performed a review of the available literature to uncover the confirmed direct targets of miR-451a and their association with RPLP1.

Materials and Methods

Cell culture and transfection assay

12Z immortalized endometriotic epithelia cells were obtained from Dr. Linda Griffith, MIT via a Materials Transfer Agreement (MTA). The endometriotic 12Z cells were seeded at density of 1×10^6 cells/ml of media in T-75 flask using complete culture media: phenol red-free Dulbecco's Minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co.) + 10% charcoal stripped FBS (Atlanta Biologicals, Atlanta, GA, USA) + Pen-Strep (Sigma Chemical Co). Once the cells reached approximately 90% confluency, the cells were passed and plated into 6 wells plates at 1×10^5 cells/ml in phenol red-free Dulbecco's Minimum essential medium (DMEM)/Ham's F12 (Sigma Chemical Co.) with no FBS and P/S added and placed in the incubator for 24h.

For miR-451a transfection, after 24h of plating the cell, 12Z cells were transfected using Lipofectamine 2000 transfection agent (Life Technologies/Ambion, Inc.) following the recommendations of the manufacturer with either Lipofectamine 2000 transfection reagent mixed with 30 nM of pre-miR451 (pre451; Life Technologies/Ambion, Inc., Grand Island, NY, USA) or a non-targeting mimic (NT-mimic, negative control; Life Technologies/Ambion, Inc.). At 24h and 48h post-transfection, RNA and proteins were collected for qRT-PCR, and western blot.

For Myc knockdown, cells were transfected using Lipofectamine 2000 transfection agent (Life Technologies/Ambion, Inc.) following the recommendations of the manufacturer. For this experiment, 12Z cells were plated at 1×10^5 of 6-well plates in DMEM: F12 with pen/strep and 10% FBS for 24h. Cells were transfected with either Lipofectamine 2000 transfection reagent mixed with 40 nM siRNA for Myc or a non-targeting (negative control) and added to 6-well plates.

The transfection was performed at 24h, and 48h after plating to maximize the transfection efficiency. At 24h and 48h post-transfection, RNA and proteins were collected for qRT-PCR, and western blot. For each treatment, cells were cultured in duplicates and then for RNA and protein analysis.

RNA isolation and qPCR

For assessment of mRNA, total RNA was isolated using the Tri-reagent (Sigma Chemical Co., St. Louis, MO, USA). Then, the total RNA (1 µg in 20 µl) was used for reverse transcription using RT kits (Applied Biosystems; Foster City, CA, USA), according to the manufacture instructions. Primer-Blast was used for primers design, and for 18S (control). The primers used in this study are RPLP1 V1: forward 5'-TGACAGTCACGGAGGATAAGA-3', reverse 5'-CCAGGCCAAAAAGGCTCAAC-3'. RPLP1 V2: forward 5'-CTCACTTCATCCGGCGACTA-3', reverse 5'-GCCAGGGCCGTGACTGT-3'. c-Myc: forward 5'-GTAGTGGAAAACCAGCAGCC-3', reverse 5'-AGAAATACGGCTGCACCGAG-3' were synthesized by Integrated DNA Technology (IDT, Coralville, IA). The resulting samples were used for qRT-PCR by using QuantStudio 7 Flex System. All samples were run in triplicate, and the calculation was done by using the average values, and the 2-delta-delta CT method. In human tissues experiment analysis, human 18S primers probe reagents were analyzed, and the fold changes were normalized to the eutopic endometrium. In 12Z human endometriotic cells experiment analysis, human 18S primers probe reagents were analyzed, and the fold changes were normalized to the non-targeting (negative control). All Samples were tested in triplicates and represented as the mean \pm SEM.

Protein Isolation and Western blot

The human tissues were homogenized in RIPA Buffer (Cell Signaling Technology, 9806) with PMSF (Cell Signaling Technology, 8553S) and total protein concentration measured using Bio-Rad by Protein Assays ([Catalog 3500-0006], Bio-Rad Laboratories, Richmond, CA, USA).

To isolate the protein from the 12Z human endometriotic epithelial cell line, the protein was isolated by using lysis buffer (Cell Signaling Technology, 9803) with PMSF (Cell Signaling Technology, 8553S), and then the Bio-Rad Protein Assay ([Catalog 3500-0006], Bio-Rad Laboratories, Richmond, CA, USA) was used to determine the total protein.

The same amount of protein (50 µg) was electrophoresed through 12% Bis (2-hydroxyethyl) amino-tris(hydroxymethyl)methane (w/v) gels and electro-blotted onto nitrocellulose membranes (Invitrogen). Primary antibodies: Myc (Protein Tech Cat# 10828-1-AP), RPLP1 (Protein Tech Cat# 216363-I-AP) and goat anti-rabbit secondary antibody (1:5000; GE Healthcare/Fisher Scientific, Pittsburgh, PA, USA) were used. To normalize the protein expression levels, stripping and re-probing for beta-actin (Abcam, Cambridge, MA, USA). The immuno-detection was performed by using enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA).

Human Tissues

We utilized tissue samples from our IRB-approved bank of specimens which were obtained from the University of Kansas Medical Center, Department of Pathology and Laboratory Medicine and/or the Department of Obstetrics and Gynecology at the Cleveland Clinic. This consists of paraffin-embedded tissue sections for immunohistochemistry studies as well as frozen tissue for protein and RNA analysis (western blot and PCR, respectively).

Samples were obtained from subjects between the ages of 21 and 45 who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue. A total of 55 subjects were enrolled (N = 23 in the proliferative stage of the menstrual cycle, N = 32 in the secretory stage of the menstrual cycle and included women with stage I/II (N = 20) and stage III/IV (N = 35) endometriosis. No subjects had taken GnRH analogs or hormonal therapy within 3 months prior to surgery. A total of 55 endometrial biopsies (eutopic endometrium) and 77 matched (same patient) endometriotic lesions were collected. All specimens were collected by the same surgeon (TF) at Cleveland Clinic or University of Kansas Medical Center (KS) with emphasis on minimizing sample contamination from underlying/surrounding non-endometriotic lesion tissue. To do so, endometriotic lesions were excised and sent to pathology for confirmation of endometriosis, which was defined as the presence of endometrial glands and stroma. Tissue was excised using sharp scissors with no energy. During the excision the underlying tissue was separated from the lesion tissue. A portion of the same sample lesion which was sent for endometriosis confirmation by a pathologist was utilized for research. Research samples were immediately snap-frozen, stored at -80 °C and then shipped to the University of Kansas Medical Center. Stage of the menstrual cycle was determined from the patient's medical records with day 1 defined as the onset of menses.

Tissue samples used as non-endometriosis controls were obtained from the University of Kansas Medical Center Department of Pathology and Laboratory Medicine. Endometrial tissue in the control groups consisted of women with uterine leiomyomas (N=12) or endometrial polyps (N=8) of which 10 samples were obtained during the proliferative stage of the menstrual cycle (N=10) and ten from the secretory (N=10) stage of the menstrual cycle. Eutopic and matched ectopic endometriotic lesion tissue (ectopic) were obtained from women with endometriosis during the

proliferative (N=12; N=4 stage I/II and N=8 stage III/IV) and secretory (N=12; N=6 stage I/II and N=6 stage III/IV) stage of the menstrual cycle. As no difference in RPLP1 mRNA, protein or miR-451 expression was noted among stages of the menstrual cycle or stages of endometriosis, data were collapsed and analyzed as ectopic versus eutopic tissue unless otherwise noted.

Statistical analysis

To test the null hypothesis that the mean levels of Myc level in ectopic endometriosis and eutopic endometriosis patients are the same, versus the alternative that the mean level in ectopic endometriosis patients is higher, a paired one-tailed t-test (0.05 significance level) was used. For correlation studies of Myc and RPLP1 V1 delta ct values in each lesion, Pearson's correlation was used. For comparison studies of the in vitro 12Z cells treatment groups, one-way ANOVA was used followed by post-analysis using Bonferroni testing or SNK procedure.

Results:

1. The expression of RPLP1 is potentially regulated through a miR-451a-Myc pathway:

The expression of RPLP1 was downregulated in 12Z cells with forced expression of miR-451a, however, based on the bio-informatic prediction tools; TargetScan 7.1, MicroRNA.org, and TargetRank, RPLP1 was not predicted to be a potential target of miR-451a, therefore, miR-451a is not expected to bind to the 3'UTR of RPLP1. Thus, we did literature review in Pubmed using the following keywords: RPLP1, ribosomal protein large P, and ribosomal protein LP1. There are only a few papers published about the functionality of this protein outside of ribosomal protein synthesis. A paper published by Qingbin and colleges [200] examined differentially expressed genes between c-Myc null and c-Myc wildtype rat fibroblast cells using cDNA micro-array analysis, and found that RPLP1 is upregulated in response to c-Myc. Based on this finding, we did another literature review and bio-informatic analysis which revealed that Myc is a direct target of miR-451a which can bind to its 3'UTR (figure 14A). Therefore, we hypothesized that miR-451a directly targets Myc which reduces the expression of RPLP1 and causes reduction in cell survival.

To test our hypothesis, we transfected 12Z endometriotic epithelial cells with 25 nM of pre-miR451a and pre-NT (negative control), and the protein and RNA were collected at 24h and 48h post-transfection to examine the level of Myc and RPLP1 by qRT-PCR and western blot, respectively. As displayed in Figure 14B, the expression level of Myc transcript was significantly lower at 24h and 48h post-transfection ($P < 0.001$; $N = 4$). Correspondingly, the expression of Myc protein was detected to be lower than the negative control at 48h post-transfection. In addition, the level of RPLP1 was examined in these cells and showed a similar

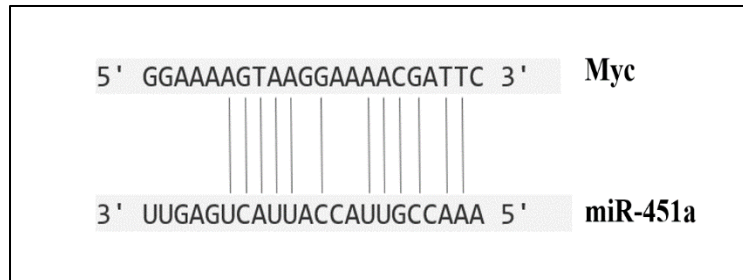
pattern of reduction (figure 6A-B). RPLP1 mRNA ($P<0.05$) and protein expression was significantly lower at 48h post-transfection, which confirmed the downregulation of miR-451a to Myc and RPLP1.

Figure 14. Over-expression of miR-451a in 12Z endometriotic epithelial cell line reduced the expression of Myc mRNA and protein. 12Z cells were transfected with pre-miR-451a (25 nm) or Pre-NT (negative control), and RNA and protein were isolated at 24 and 48h post-transfection for RNA and protein analysis.

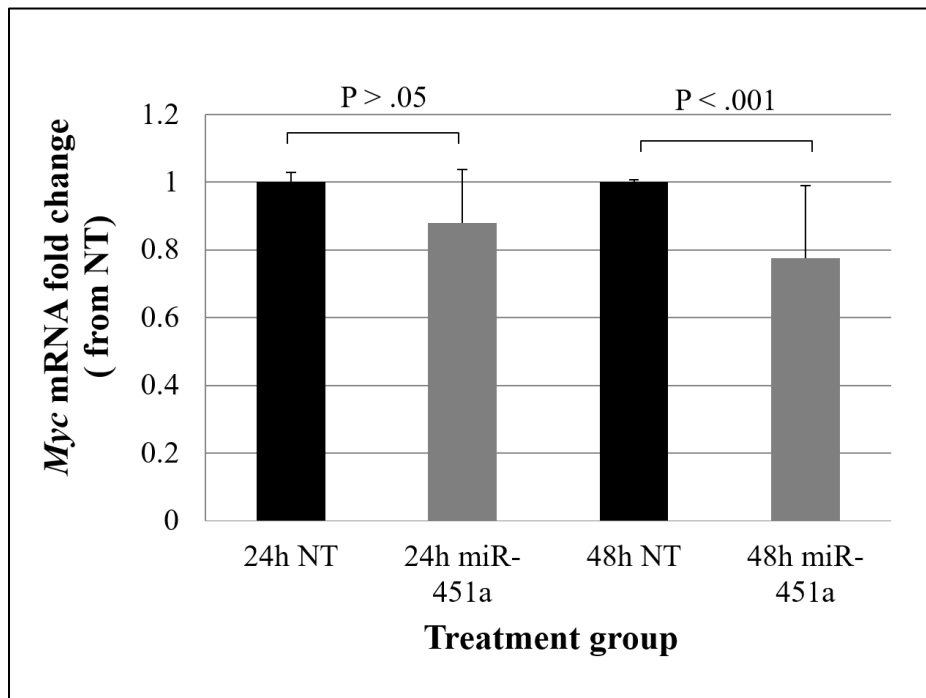
- (A) miR-451a binding sites to Myc 3'UTR was predicted by MIRANDA
- (B) qRT-PCR analysis of Myc mRNA demonstrated a reduction in the level of Myc transcript in 12Z cells transfected with pre-miR-451a compared to negative control cells, at 24h and 48h post-transfection (The expression of Myc was normalized to 18S). (At 48h: $P < 0.001$; $N = 4$). Data were analyzed by one-way ANOVA followed by post-hoc analysis using Bonferroni testing. All data are displayed as the mean fold change from 24h NT \pm SEM ($N = 4$).
- (C) Western blot of the cell lysates showed a downregulation of Myc protein in 12Z cell transfected with pre-miR-451a compared to non-targeting cells at 48h post-transfection. (Beta-actin used as a loading control).

Figure 14

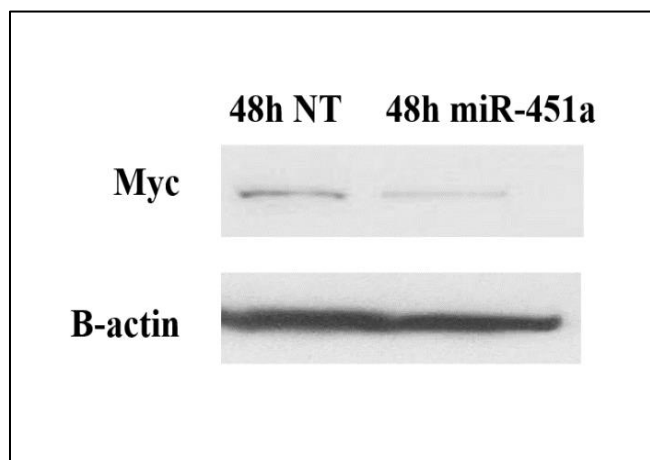
A



B



C



2. Myc deletion reduced the expression of RPLP1 in 12Z endometriotic epithelial cell line:

To confirm that the expression of RPLP1 is regulated by Myc, we knocked down the expression of Myc by transfecting 12Z cells with siRNA for Myc or non-targeting siRNA (negative control) at 24h and then did another transfection at 48h. The RNA and protein were collected at 24h and 48h following the double transfection to analyze the level of Myc and RPLP1 transcript and protein by qRT-PCR and western blot. As shown in figure 15A and 15C, the expression levels of Myc mRNA and protein were significantly reduced at 24h and 48h compared to the negative control ($P < 0.04$; $N = 4$), which confirms that the knockdown was successful. This reduction was associated with the downregulation of RPLP1. As depicted in figure 15B, the expression level of RPLP1 transcript was dramatically reduced at 24h and 48h as a result of Myc deletion compared to the negative control ($P = 0.05$; $N = 4$). In addition, RPLP1 protein was shown to be reduced in 12Z cells transfected with siRNA for Myc at 24h and 48h post-transfection, compared to the non-targeting cells (Figure 15C). These findings suggest a role for Myc in regulating the expression of RPLP1.

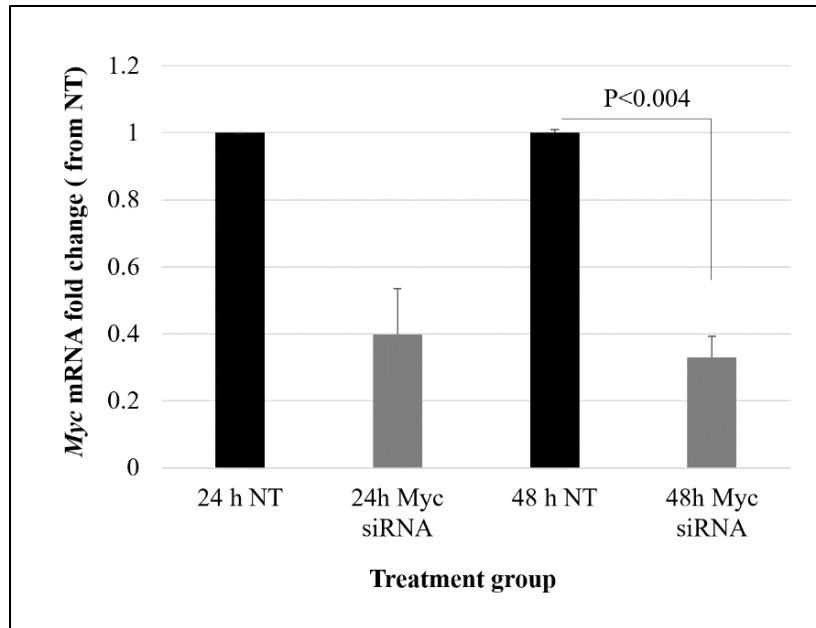
Figure 15. Myc Knockdown reduced RPLP1 expression. Immortalized human endometriotic epithelial cell line (12Z) were transfected with siRNA for Myc, or non-targeting siRNA (negative control) at 24h and 48h, RNA and protein were isolated after each time points (n=4). The expression is represented as the fold change from control (non-targeting) \pm SEM.

(A) qRT-PCR analysis of Myc and RPLP1 variants (V1 and V2) expression (B) at 24h and 48h post-transfection demonstrated a reduction following the transfection with siRNA for Myc. Data were analyzed by one-way ANOVA followed by post-hoc analysis using Bonferroni testing. All data are displayed as the mean fold change from 24h NT + SEM (N=4).

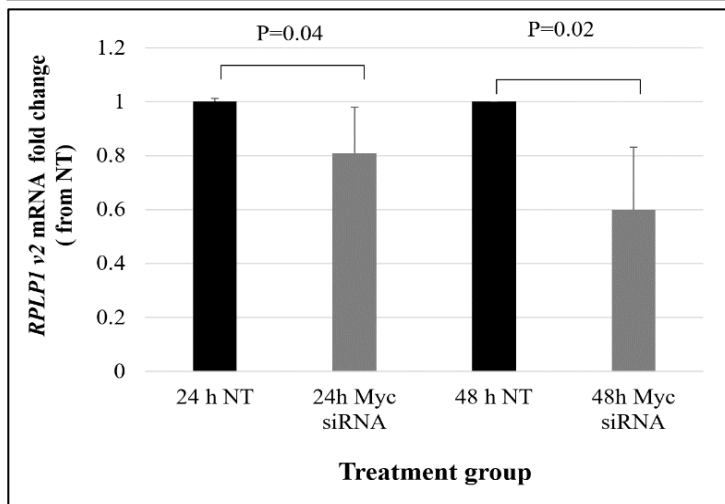
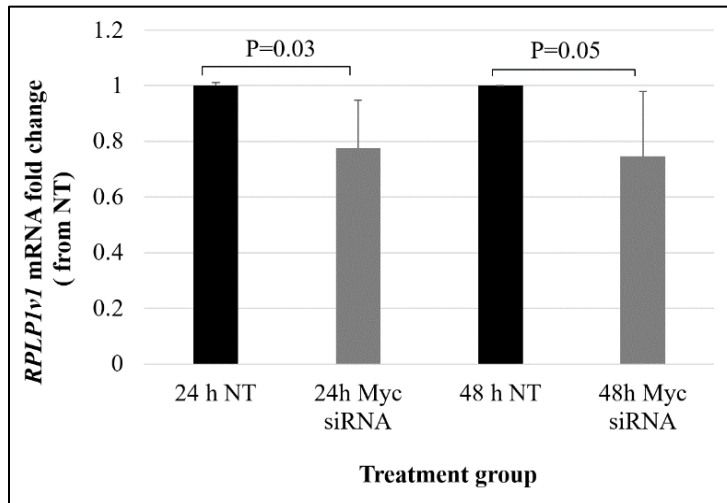
(C) Western blot of Myc and RPLP1 of cell lysates, which showed downregulation in 12Z cells with Myc knockdown.

A

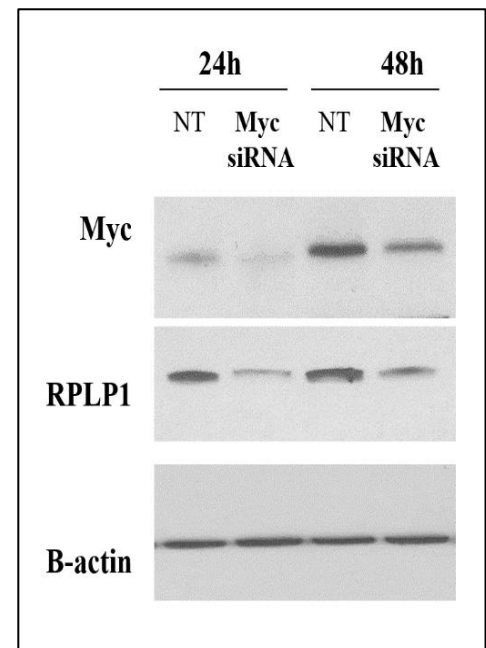
Figure 15



B



C



3. Myc mRNA and protein is over-expressed in human endometriotic lesions and positively correlated with the expression of RPLP1:

To confirm that Myc controls the expression RPLP1 in human endometriotic lesions, we first measured the level of Myc mRNA by qRT-PCR, in matched eutopic and ectopic tissue from women with endometriosis. For this analysis, we used human tissues (eutopic endometrial tissue from women with endometriosis, N=55, and matched ectopic lesions, N=77) from the Cleveland Clinic, Department of Obstetrics and Gynecology. Myc expression was not influenced by stage of menstrual cycle (proliferative versus secretory) or stage of endometriosis (I/II versus III/IV) so data were analyzed as eutopic versus ectopic endometrial tissue. As displayed in figure 16A, the fold change of expression of Myc mRNA was higher (>1.0) in 70% (54/77) of the ectopic lesions compared to matched eutopic tissues. Further, 30% (23/77) of the ectopic lesions showed lower expression of Myc transcripts (≤ 1.0) compared to matched eutopic tissues. The overall mean fold change of expression of Myc transcript was significantly higher in ectopic lesion compared to eutopic tissues (4.1-fold increase; $P < 0.001$) as shown in figure 16B. The expression of RPLP1 and Myc transcripts was assessed and compared in the same sample, which shows that the level of expression of RPLP1 and Myc was positively correlated (Pearson $r = 0.56$; $P < 0.01$) (figure 17A). All in all, these data confirm our hypothesis of the association of Myc and RPLP1.

Figure 16. Total expression of Myc transcripts is elevated in endometriotic lesions compared to matched eutopic tissues.

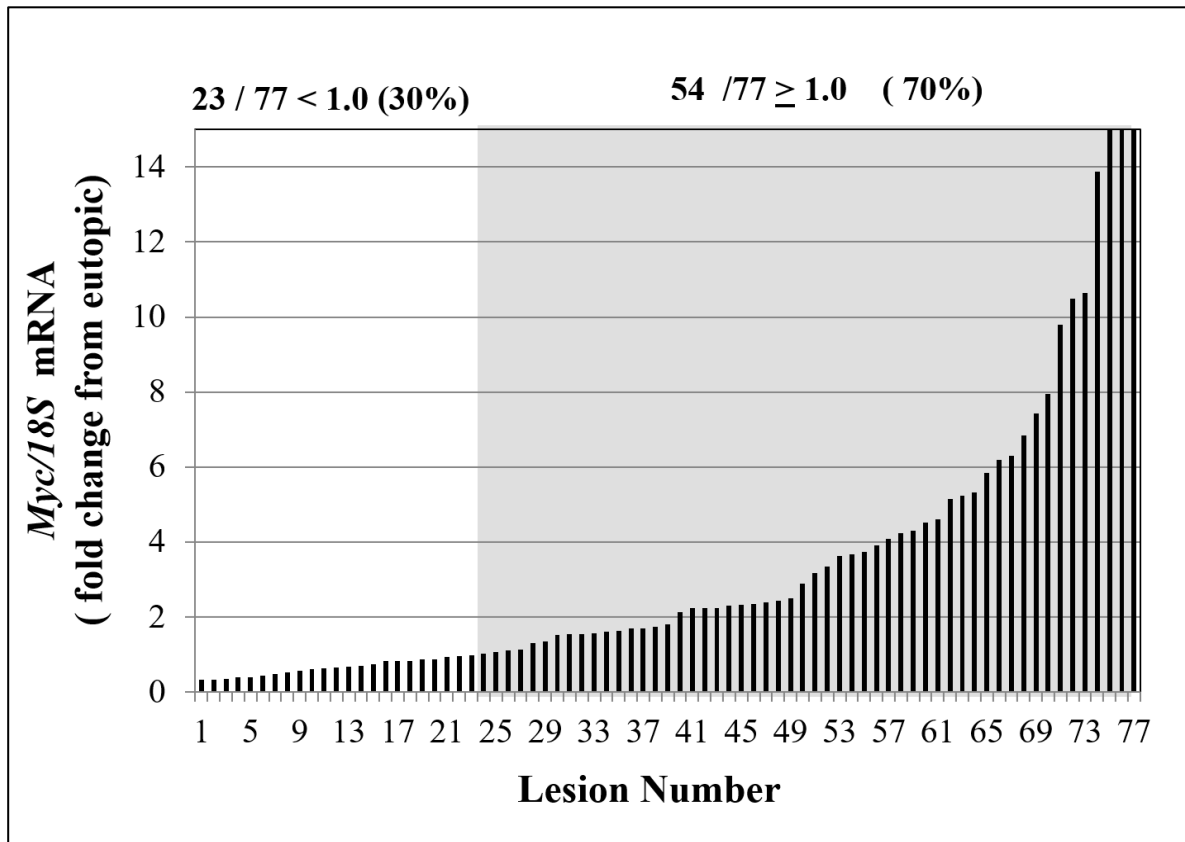
(A) qRT-PCR analysis of Myc mRNA in endometriotic lesions showed an elevation of the fold change of expression of Myc (>1.0) in 54 / 77 (70%) and downregulation (≤ 1.0) in 23 / 77 (30%) of the endometriotic lesions compared to matched eutopic tissues. Data were analyzed by unpaired t-test and are displayed as the mean + SEM fold change from eutopic endometrium (N=55 eutopic endometrium, N=77 matched ectopic lesions).

(Note that sample numbers 75 -77 were capped at a 15-fold increase in this figure; actual fold increases for samples 75, 76 and 77 were 23.27, 32.72, 52.45-fold higher, respectively.)

(B) qRT-PCR analysis of Myc transcript in endometriotic lesions represented by the mean fold change of expression of Myc demonstrated to be increased in ectopic lesions compared to eutopic tissues. (4.1-fold increase; $P<0.01$)

Figure 16

A



B

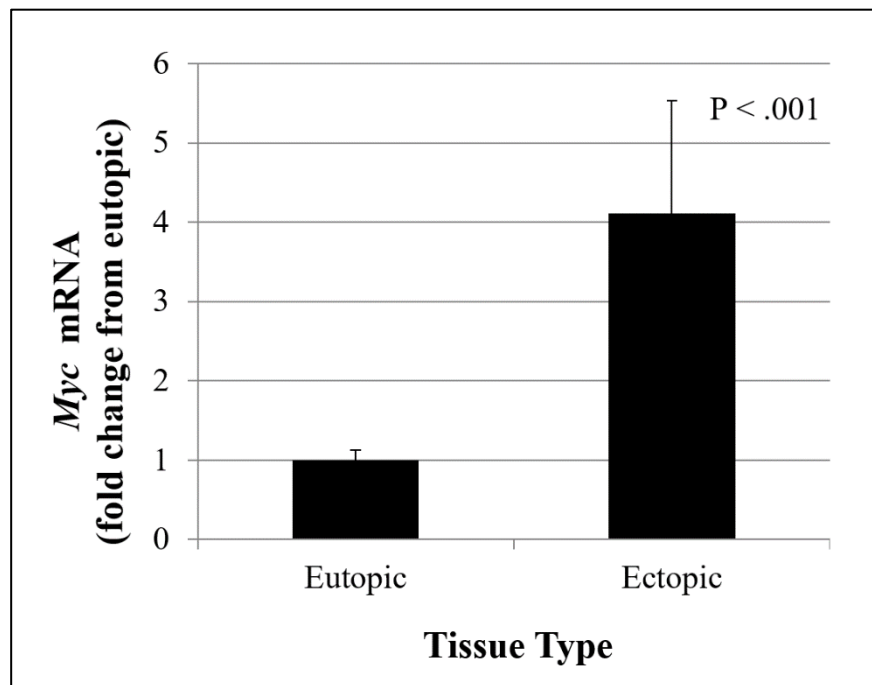
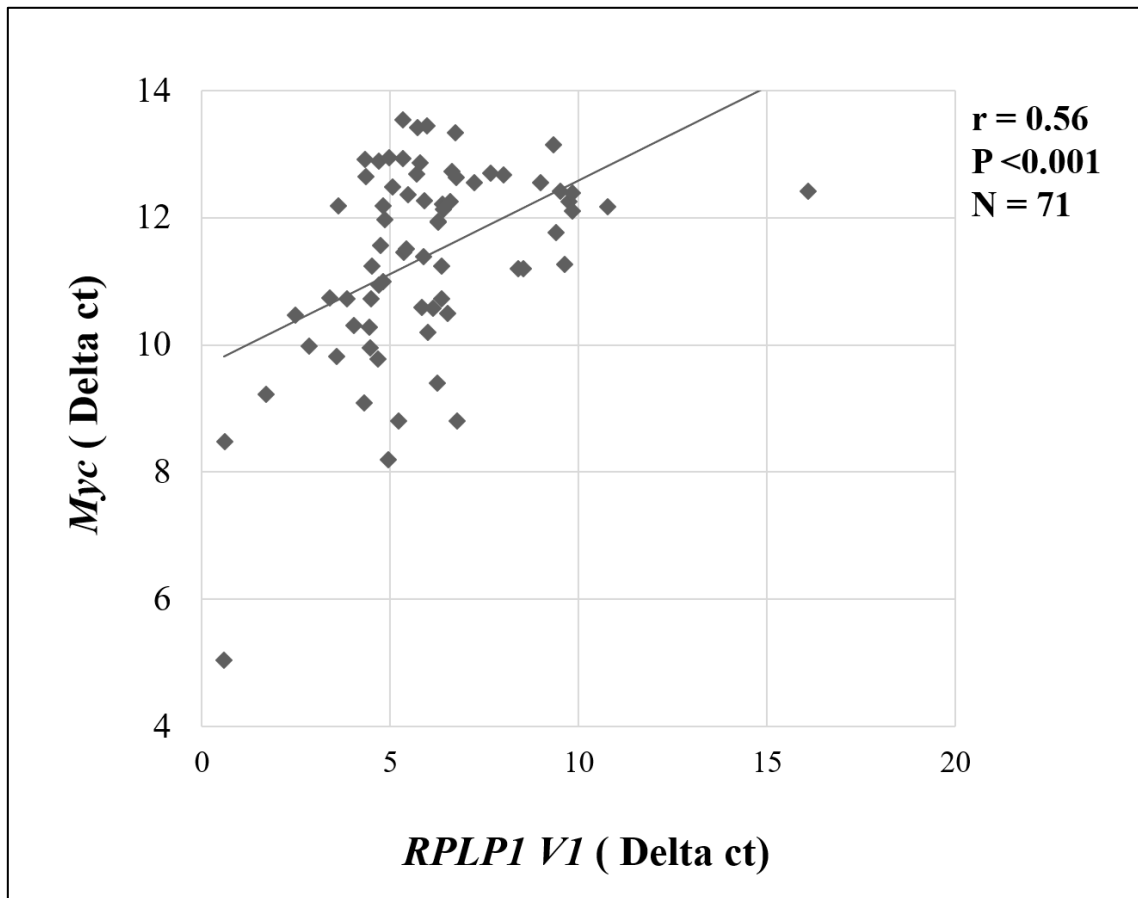


Figure 17. The expression of Myc is positively correlated with RPLP1 in human endometriotic lesions.

- (A) The data represented by Delta ct values for Myc and RPLP1 in the same sample. Lower delta ct values correspond to a higher level of mRNA expression. Endometriotic lesions which express high levels of Myc (lower left corner of graph) also expressed high levels of RPLP1, while lesions which express lower levels of Myc mRNA (upper right corner of graph) express lower levels of RPLP1 mRNA. Overall assessment of 71 total lesions revealed a positive correlation between transcripts RPLP1 (Pearson $r = 0.56$; $P < 0.001$). Myc and RPLP1 data were normalized to the level of 18S transcript, and represented as the fold change from control (eutopic tissues) \pm SEM.

Figure 17

A



Discussion:

To expand our knowledge on the expression and role of miR-451a and RPLP1 in endometriosis pathophysiology, we wanted first to determine if RPLP1 is a direct target of miR-451a. For this purpose, we used target predictions resources including TargetScan 7.1, MicroRNA.org, and TargetRank. These tools suggested that RPLP1 is not a potential direct target of miR-451a. Therefore, we hypothesized that RPLP1 is a potential indirect target of miR-451a, through an alternative mechanism or pathway allowing miR-451a to control the expression of RPLP1.

According to our data, it is tempting to classify endometriosis into ribosomopathies considering our finding of ribosomal protein dysregulation. However, it should be noted that RPLP1 and RPLP2 are located free in the cytoplasm and only form the ribosomal stalk to participate in protein translation [209]. Hence, RPLP1 may gain its function in driving cellular and lesion proliferation by interacting with other factors or proteins in its cytoplasmic free state, and not from its role in ribosomal structure. Supporting this hypothesis, a previous study conducted to investigate the effect of ribosomal proteins knockdown, including RPLP1, on ribosome function and structure which demonstrated that the number and function of ribosomes were not decreased upon RPLP1 reduction and the total expression profiles were not compromised [180].

There was limited available research focusing on the function of RPLP1 in pathological conditions; one study conducted by Guo and colleagues found RPLP1 as a c-Myc responsive gene [200]. Myc (*v-myc* myelocytomatosis viral oncogene homolog) is a transcriptional factor that dimerizes with another protein, Max, and binds to the E-box sequence of the promoter sequence and controls the expression of downstream genes. It regulates the expression of 15% of genes in human [210] and controls several genes responsible for proliferation, tumorigenesis, and apoptosis [211-214]. The

mis-expression of Myc was defined as a “hallmark” of cancer [215] and it is detected in several types of cancer including breast [216], lung [217], ovarian cancer [218] and prostate cancer [219]. Furthermore, dysregulation of Myc was also identified in inflammatory and autoimmune diseases such as rheumatoid arthritis [220], and chronic liver disease, and found to stimulate the expression of inflammatory factors [221].

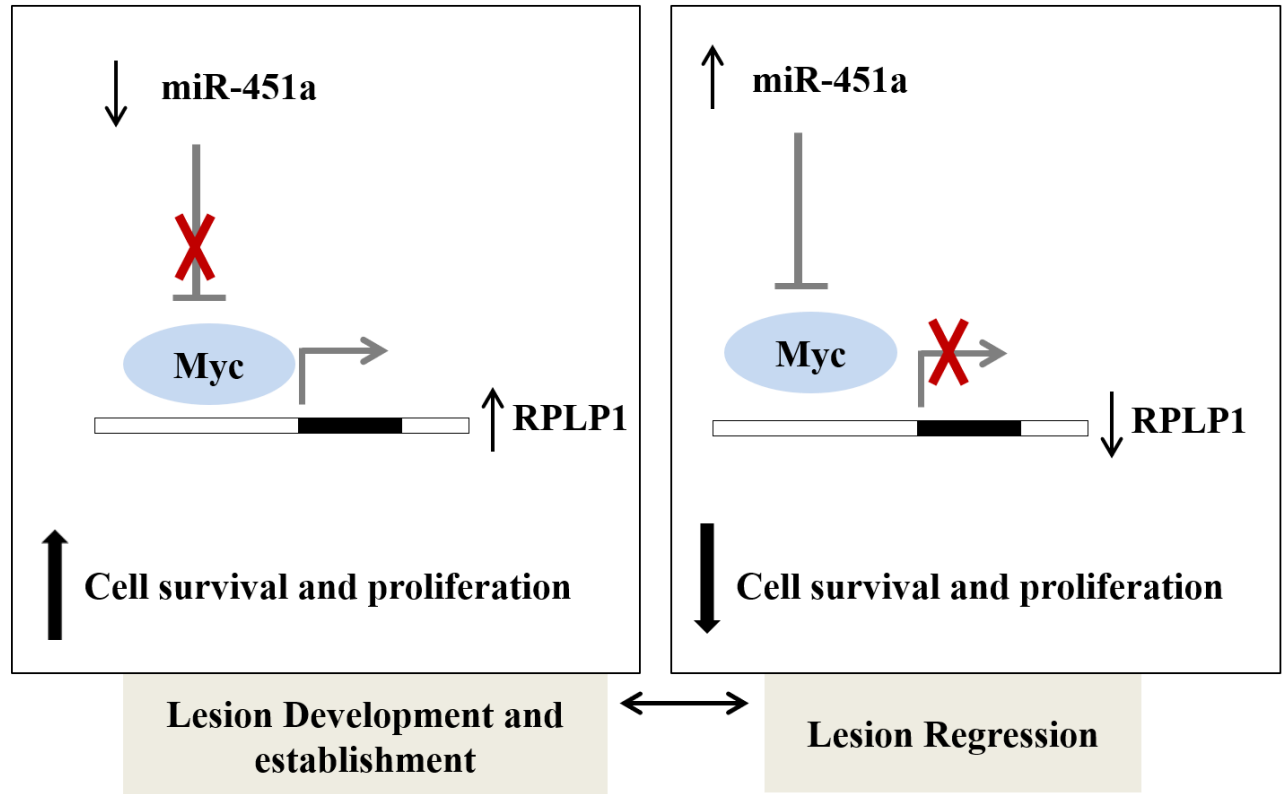
In cancer cell lines, several studies showed that Myc is a direct target of miR-451a, and this was confirmed at the RNA and protein levels, as well as through the use of reporter assays [157, 160-162]. In endometriosis, the expression of Myc was found to be highly expressed in the epithelial ectopic lesions compared to eutopic tissues and associated with proliferation [222]. In human endometriotic cyst stromal cells (ECSCs), c-Myc was found to be suppressed by miR-196b [223]. Further studies indicated the up-regulation of Myc in endometriotic lesions compared to eutopic endometrium from women with or without endometriosis [124, 182, 224-227]. However, most of these were descriptive studies to show the differential expressed proteins profile and have not investigated the proliferative mechanism of Myc in endometriotic lesions. Based on this, we hypothesized that miR-451a may directly bind to Myc, which controls the expression of RPLP1 and modulates proliferation.

The expression of c-Myc in the human samples was significantly elevated in ectopic lesions compared to eutopic tissues, and positively correlated with the expression of RPLP1. Furthermore, our in vitro study confirmed that c-Myc is a potential direct target in 12Z cell line, this is demonstrated by reduction of c-Myc RNA and protein levels as a result of miR-451a over-expression. The knockdown of c-Myc caused a significant decrease in RPLP1 expression, which indicated a possible regulation of RPLP1 by c-Myc.

Based on our understanding of the endometriotic lesion pathophysiology, which is expected to go through cycles of regression and proliferation depending on hormonal and other unknown factors (figure 18), we propose that miR-451a may regulate lesion survival by directly repressing Myc, which in turn reduces the expression of RPLP1. Because of RPLP1's role in proliferation, its downregulation leads to a reduction in cell survival and proliferation. On the other hand, low levels of miR451a in the lesion allows expression of Myc and, therefore, increases the expression of RPLP1 which induces lesion development and survival.

Figure 18. The pathway of miR-451a in modulating lesion proliferation through targeting c-Myc-RPLP1.

Figure 18



Chapter IV:

Matrix metalloproteinases and endometriosis: Their Role in Disease pathophysiology and potential as therapeutic targets [228]

This chapter is reprinted with permission. Z. Alali, K. Swan, W.B. Nothnick, Matrix Metalloproteinases and Endometriosis: Their Role in Disease Pathophysiology and Potential as Therapeutic Targets, Current Women's Health Reviews 13 (2017) 1-7.

Abstract

Background: The matrix metalloproteinase (MMP) system is a group of enzymes, which function to modulate the tissue structure and degrade the extracellular matrix (ECM), a process required in cellular repair, proliferation, apoptosis, and angiogenesis. The role of MMPs in endometriosis pathophysiology has been examined, and it is hypothesized that mis-expression of MMPs in endometrial cells or surrounding tissues is a key factor in promoting the attachment, invasion, and angiogenesis required for establishment of ectopic lesions. **Objective:** The objective of this review is to update the current state of knowledge on the role of MMPs in the pathophysiology of endometriosis and discuss the potential utility of treatments that may directly or indirectly target their action in endometriosis. **Results:** In this review we summarize the current state of knowledge on the MMPs and the pathogenesis of endometriosis, discuss the role of the MMPs in endometriosis pathophysiology, summarize current treatments for endometriosis and discuss potential utility of inhibition of MMP action in endometriosis treatment. **Conclusion:** Based upon the current state of knowledge, therapeutic approaches targeting MMPs may be useful in mitigating the proliferation and the establishment of the lesions, but further patient-based studies are clearly needed.

1. Introduction

Endometriosis is estrogen-dependent disease which is defined as the presence of ectopic endometrial tissue primarily within the pelvic cavity. Endometriosis is characterized primarily by pelvic pain, and infertility. The factors which lead to the initial development, progression and survival of these ectopic lesions are poorly understood. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are involved in several biological functions including, tissue repair and remodeling. Normally, MMPs play a role in modulating the extracellular matrix and tissue remodeling which occurs within the endometrial lining during the menstrual cycle. MMP expression and activity is proposed to be altered in endometriotic lesion tissue as a result of dysfunctional steroid and cytokine expression and/or action. In turn, altered MMP expression/activity is proposed to enhance the invasion, attachment and proliferation of the ectopic lesion. In this review, we will highlight the potential role of the MMP system in the pathogenesis of endometriosis with emphasis on recent therapy targeting MMPs and their biological activity.

2. The Matrix Metalloproteinase System

The matrix metalloproteinase (MMP) system is a group of enzymes (the MMPs) and their enzyme tissue inhibitory regulators, the tissue inhibitors of metalloproteinases (TIMPs), which plays a role in numerous biological functions associated with extracellular matrix turnover and tissue repair. The major function of MMP enzymes is to degrade extracellular matrix proteins leading to tissue remodeling and repair, apoptosis, angiogenesis, cellular proliferation and invasion. MMPs are zinc-containing endopeptidases, which share the same structural domains; the pro-peptide, the catalytic domain, hinge region, and haemopexin-like C-terminal domain. Currently, there are 26 MMPs found, which have been classified according to their cellular localizations or specificity [229].

MMPs classes include, collagenase (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinase (MMP-2, and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-27), matrilysins (MMP-7, and MMP-26), membrane type MT-MMP (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), enamelysin (MMP-20), macrophage metalloelastase (MMP-12), and other enzymes (MMP-19, MMP-21, MMP-22, MMP-23A, MMP-23 B, MMP-28, and MMP-29). MMPs are mostly secreted proteins with exception of the membrane type, MT-MMPs, which anchor to the cell membrane. Normally, these enzymes are regulated in three stages, the first stage requires the transcription of these proteins, while the second stage involves the conversion of MMPs from the inactive zymogens form to the active form under the action of other proteases. Lastly, the MMPs are controlled at the tissue level by the TIMPs and the ratio of MMPs to the TIMPs must be tightly controlled [230].

MMPs are released by many cells' types, including endothelial cells, fibroblasts, and immune cells such as macrophages and neutrophils, and involve the regulation of various physiological processes. For example, the reproductive system is subjected to various structural changes in tissues architecture during the processes of ovulation, menstruation, and embryo implantation. MMPs have been investigated thoroughly as a significant player in these events. For instance, MMPs are postulated to enhance the ovulation process by breaking down the collagen components of the ovarian follicles, leading to its rupture and oocyte release [231]. Within the uterus, MMP-7 mRNA has been associated with the endometrial epithelial cells in menstruation and tissue repair [232, 233]

The regulation of the activity of MMPs is essential to avoid disturbances in the normal balance in their levels, which could result in several pathological manifestations. MMPs are expected to be regulated by hormonal or cytokines stimuli, such as Interlukin-1 (L-1), and tumor necrosis

factor (TNF- α). Specifically, TNF- α is produced by macrophages due to injury or stress. TNF- α can bind to the membrane receptors on the targeted cell and stimulate the expression of MMPs. As a consequence, imbalance in the expression levels of MMPs, TIMPs, or regulatory factors is associated with some types of cancer and inflammation. For instance, increases the level of TNF- α in peritoneal cavity of women is consider a promoting factor to increase the MMPs leading to endometriosis, a gynecological inflammatory disease [234].

3. The Pathophysiology of Endometriosis

Endometriosis is an estrogen dependent disease characterized by the accumulation of endometrial stroma and glandular tissues ectopically, mostly on ovaries, fallopian tubes, and peritoneal lining of the pelvic cavity. The disease is primarily associated with pelvic pain, dysmenorrhea, and infertility. It affects around 10 % of women in reproductive age, with annual medical care cost of approximately \$ 20 billion in United States [235]. Several theories have been postulated which propose mechanisms, origins and pathogenesis of endometriosis. The coelomic metaplasia theory suggests that the ectopic endometrial tissue is transformed from peritoneal mesothelium. The transformation of the tissues is explained to be enhanced by steroidal , and immunological stimuli [236]. A more recent theory was based on the observation that self-renewal adult stem cells/progenitors are located within the endometrium, which suggests a potential differentiation of these stem cells into endometrial cells and migration ectopically [237].

To explain the observation of endometriosis in female embryos, it is proposed that the endometrial cells outside of the uterus is generated originally from imperfect growth of Wolffian or Müllerian ducts during embryo development. This theory speculated that ectopic endometrial

lesion is developed as a result of the existence of Wolffian or Mullerian ducts cells remnants which progress into estrogen-responsive lesion [110].

Retrograde menstruation is another theory which suggests that retrogradely shed endometrial fragments migrate through the fallopian tubes and attach to pelvic cavity organ surfaces establishing vascularization and eliciting estrogen responsiveness [235, 238, 239]. Although the theories explain the existence of endometrial cells in the ectopic locations, the rationales for the ability of these tissues to survive, invade, and avoid the immune response is still indefinite. Accordingly, endometriosis is also hypothesized to be a chronic inflammatory disorder caused by dysfunction or imbalance in the immunity components.

Supporting this hypothesis is the observation that women with endometriosis show high levels of activated macrophages and low natural killer cells [240]. A variety of cytokines have been shown to be highly expressed in the peritoneal fluid of women with endometriosis, giving more evidence of the inflammatory nature of the disease [241]. TNF- α , in particular, is a pro-inflammatory cytokine which is elevated in the peritoneal fluid and sera of women with endometriosis and it is positively associated with the severity of the disease [242-244]. In addition, it is suggested that TNF- α polymorphism is a possible cause for the pathogenesis of endometriosis [245, 246]. TNF- α is proposed to enhance the proliferation of the endometriotic lesion while elevated level of peritoneal fluid TNF- α is proposed to introduce a toxic environment that reduces oocyte quality resulting in fertilization or implantation difficulties [247, 248]. The mechanism of action of TNF- α in endometriosis is postulated to be through stimulating the secretion of numerous components including MMPs, which help to degrade the extracellular matrix allowing the invasion of the lesions into the mesothelial cells of the peritoneal lining [249].

4. The Role of The MMP System in The Pathophysiology Of Endometriosis

In healthy women, the expression of MMPs fluctuates in a menstrual cycle-dependent fashion. MMP expression is elevated during the period of menstruation to regulate the degradation and shedding of endometrium, and in proliferate phase to guide the repairing process which occurs during regeneration of the endometrial lining. However, the expression of MMPs is more tightly controlled during the secretory stage of the menstrual cycle, particularly during the window of implantation [250]. In endometriosis patients, it has been found that the expression level of specific MMPs is higher in ectopic endometriotic tissues lesions compared to eutopic lesion suggesting a potential role of MMPs in stimulating the onset and the establishment of the disease. To date, studies have shown the elevation or mis-expression of MMPs and TIMPs in endometriosis patients, such as MMP-1[251, 252], MMP-2[253], MMP-3[254, 255], MMP-7[256, 257], MMP-9 [258], MMP-13 [259], MMP14 (MT1-MMP) [253], MMP-24 (MT5-MMP) [260], TIMP-2[252], and TIMP-3 [258] (Table 1).

Due to the heterogenicity nature of the endometriotic tissues, and depending on the sample/biopsy under investigation; levels of MMPs and their inhibitors (TIMPs)

Table 1. Differential expression levels of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in human endometrium/endometriotic lesion tissue.

MMPs/TIMPs	Study findings [reference]
MMP-1	MMP-1 mRNA expression was detected in red peritoneal and ovarian endometriosis [24]
MMP-1 TIMP-1 TIMP-2	MMP-1 protein is elevated and TIMP-1 and-2 proteins are reduced in endometriotic tissue compared to endometrium [25]

MMP-2 MT1-MMP TIMP-2	MMP-2 and MT1-MMP are up-regulated, and TIMP-2 is downregulated in eutopic endometrium from patients with endometriosis compared to endometrium from endometriosis-free women [26].
MMP-3	MP-3 mRNA is elevated in peripheral blood of endometriosis patients compared to patients without endometriosis [28].
MMP-7	MMP-7 is differentially expressed in endometriosis depending on the types of endometriotic lesions [30].
MMP-9 TIMP-3	TIMP-3 is reduced in eutopic endometrium and ectopic endometriotic tissue compared to endometrium of women without endometriosis. MMP-9 and MMP-9/TIMP-3 ratio is elevated in ectopic tissues compared to endometrium from women with and endometriosis [31].
MMP-9 TIMP-1	MMP-9 transcript is increased in eutopic endometrium of endometriosis patients compared to endometrium of control women. TIMP-1 transcript is decreased in ectopic lesion compared to eutopic endometrium of patients with endometriosis and control women [34].
MMP-9	MMP-9 is elevated in the plasma and peritoneal fluid in endometriosis patients compared to endometriosis-free women [37].
MMP-13 MMP-14 MT1-MMP	The abundance of active MMP-13 and MT1-MMP / MMP-14 are reduced in peritoneal fluid of endometriosis patients compared to women without endometriosis [32].
MT5-MMP	MT5-MMP level is increased in eutopic endometrium and ectopic peritoneal lesions of women with endometriosis [33].

could vary. For instance, TIMP-1 transcript was lower in human ectopic endometriotic lesion compared to matched eutopic endometrium and to endometrium from women without endometriosis [261]. On the contrary, recent proteomic studies to examine the protein profile of cervical mucus of endometriosis patients demonstrated that the expression of TIMP-1 is

significantly higher in cervical mucus of patients with endometriosis compared to endometriosis-free women [262].

Evaluating the factors involved in activation or inhibition of MMPs is important to understand how MMPs contribute to the pathogenesis of endometriosis. A study was conducted to examine the level of MMP-9 in the serum, ascites, and endometriotic lesions in patients with confirmed endometriosis compared to control group. In this study the severity of the disease (endometriosis lesion staging), and the phase of menstrual cycle were documented. Elevated levels of MMP-9 were detected in the serum and ascites of endometriosis patients compared to disease-free group. In the endometriosis patients group, it was found that MMP-9 is higher in ectopic endometriotic lesions compared to the eutopic endometrium, and that this elevation was more prominent in the proliferative stage and in the advanced stage of the disease than in subjects with less invasive stages [263, 264]. These results were interpreted to suggest a possible correlation between the severity of the disease and the pattern of MMP expression. Further, a prior study demonstrated a positive correlation between an increased ratio of MMP-9 to TIMP-1 with endometriosis pathogenesis [265].

Angiogenesis is an essential step in establishing and maintaining the ectopic lesion. To examine the factors controlling this process in endometriosis, Jana and colleagues examined the effect of MMP-2 and COX-2 inhibition on angiogenesis of the endometriotic lesion using an experimental mouse model for endometriosis. They found that there was a reduction in numbers of lesions formed in mice treated with inhibitors compared to the control mice. *In vitro*, the study showed decrease cellular migration and tubal development due to MMP-2 inhibition which suggests potential role of MMP-2 in angiogenesis. The functions of MMP-2 in sustaining the

invasiveness and angiogenesis of the disease has shown to be regulated through COX-2–PGE2-pAKT pathway. [266].

It is well-established that endometriosis is an estrogen-dependent and progesterone-resistant disease. *In vitro*, endometriotic cells show increased proliferation and growth following estrogen treatment, and lesion regression after progesterone supplement. These cyclic changes in endometrial tissues coincide with the expression level of MMPs which increases during the proliferative phase of the cycle and decrease during the secretory phase. Accordingly, the effects of estradiol treatment on the expression and the activity of MMPs have been investigated in several studies. Immunohistochemical analysis comparing the expression of MMP-1, ER- β , and ER- α in human endometriotic specimen to matched samples of endometrial tissues from the same patients, and to endometrium from healthy controls demonstrated a correlation in the expression of ER- β and MMP-1 in the ectopic lesion, in which both of these were elevated. However, ER- α expression levels were reduced in the same tissues. This comparative analysis proposes potential involvement of MMP-1 in inducing endometriosis through an estrogen dependent pathway [267].

An *in vivo* study to investigate the role of steroid hormones in the MMPs expression was conducted by Wang and colleagues in which endometriosis was induced in immunocompromised mice using human endometrial tissue. Mice were treated with estrogen, progestin, estrogen and progestin, and saline. The isolated lesion from estrogen and estrogen plus progestin groups showed high expression of MMP-2 and low TIMP-2. In addition, TIMP-2 was lower in the progestin treated group. The authors concluded from this study that the cyclic hormonal changes increased the ratio of MMP-2 to TIMP-2 to allow the establishment of endometriotic lesions [268].

MMPs have also been examined based upon the inflammatory nature of endometriosis and its association with immune dysfunction and imbalance. It was found that the peritoneal cavity of endometriosis patients contains excessive level of TNF- α which stimulates the production of MMPs and the adhesion and attachment of the ectopic lesions. Furthermore, TNF- α acts to increase the expression of other cytokines that further stimulate lesion establishment and survival. For example, TNF- α is postulated to elevate the synthesis of macrophage migration inhibitory factor (MIF), which is highly expressed in eutopic tissues, and the active ectopic lesion of endometriosis patients and is involved in promoting the survival and establishment of the disease [269, 270]. The role of MIF in the pathogenesis of endometriosis was examined in a study using immunocompromised mice harboring human endometrial tissue derived lesion treated with the MIF antagonist, ISO-1. Lesions isolated from ISO-1-treated mice displayed decreased levels of MMP-2 and MMP-9 which was associated with a decrease in lesion size, invasiveness, and survival [271]. This result suggested that MMPs may stimulate the migration and the severity of the disease through the same pathway as MIF. In addition, this study supports the hypothesis that endometriosis may be caused by a disturbance in the anti-inflammatory components leading to lesions escape from the host immune response. Collectively, these data have indicated strong association of MMPs and the pathogenesis of endometriosis either through estrogen mediated mechanisms as well as inflammatory pathways.

6. Potential Targets in Developing New Treatments for Endometriosis

6.1 Current treatments for endometriosis

Endometriosis treatment depends on the fact that endometriosis is a hormonal-dependent disease. Consequently, most of the available medications aim to inhibit the production or the action of steroidal hormones such as oral contraceptive (OCPs), aromatase inhibitors, and GnRH

analogues. Alternatively, in severe stage of endometriosis when the available treatments fail to improve patient conditions, laparoscopic surgery is performed to remove the ectopic lesions. However, the surgical intervention is not the best approach for all patients especially for patients who have severe widespread endometriosis, because it may result in partial or complete hysterectomy to eradicate the disease. Controlling endometriosis through medications brings more hope for patients to improve quality of life and continue their reproductive life [44, 45].

Due to low cost and availability, combination of estrogen/progestin contraceptives and progestogens are used as the primary treatment options for endometriosis patients. These hormones, such as medroxyprogesterone (DMPA) and ethinyl estradiol, act in inhibiting ovarian function and subsequently in alleviating the pain associated with endometriosis [46-48]. However, there are considerable undesirable side effects including preventing pregnancy, depression, nausea, and weight gain, which make contraceptives less-favorable for some patients [49]

Similarly, Dienogest (19-nortestosterone derivative) is progestin oral contraceptive that is used for endometriosis treatment. Dienogest has high affinity to PR (progesterone receptor), reduces the level of estrogen, and acts as antiandrogenic. In addition, Dienogest reduced the inflammation and the proliferation of endometriotic cells [50, 51]. Because of these features, the side effects associated with Dienogest administration are considerably lower than other available medications [52].

After all the surgical and medical treatments are exhausted, aromatase inhibitor (AI) is another treatment option that is suggested to be used. Aromatase is an enzyme that stimulates the conversion of steroidal precursors to estrogen. Therefore, inhibiting aromatase activity by AI reduces the level of circulating estrogen which regresses endometriotic implants and lessens

associated pain [53]. Administration of this medication alone is linked to adverse effects. To limit these risks, it is recommended to use AI in a cocktail regime with other hormonal therapy such as OCPs or GnRH analogues for endometriosis treatment [54, 55].

Gonadotropin - releasing hormone (GnRH) analogs, which block the release of gonadotropins hormones and ovarian estrogen production, are a common treatment for endometriosis. The reduction in estrogen level limits the growth and the survival of the ectopic lesion and suppresses the disease-associated symptoms. Yet, the hypo-estrogenic state introduced by GnRHa initiates some side effects such as reduced bone density, hot flashes, sweating, psychological disturbances; anxiety and depression. These associated outcomes arise because estrogen is necessary in various physiological processes other than uterine development. As a result, “add-back” therapy is followed to avoid these symptoms; a recent comparative study show that administration of GnRHa together with estradiol valerate and norethisterone acetate can mitigate the unfavorable effects and decrease the bone loss [56].

Interestingly, there is indirect evidence that the above common treatments for endometriosis may elicit their beneficial effects by acting upon cytokines and possibly MMP action. For example, GnRHa is expected to lessen the circulating peritoneal cytokines, possibly through estrogen reduction mechanisms [272]. It was also found that treatment of endometriosis patients with ultra-long GnRHa before IVF reduced the TNF- α cytokine in the follicular fluid, which lead to higher implantation and pregnancy rate [273]. Lastly, another study revealed that GnRHa may mediate a significant regression in the inflammatory nature of the peritoneal microenvironment in women with endometriosis [274]. These observations may suggest that it might be more efficient to target the ectopic implants by focusing on the inflammatory nature of the disease and aim to limit the activity of the cytokines to control the endometrial cells survival and invasion.

Finding a cure for endometriosis is important not only to control the common symptoms of the disease, but also to improve the quality of life of endometriosis patients. Few studies have linked endometriosis with the onset of depression and anxiety [32]. This may be attributed to the chronic pain associated with the disease. A study conducted to compare the psychological stress and depression in three groups: endometriosis patients with pain, pain-free patients, and control group with no endometriosis. The study showed that the quality of life and the psychological conditions were compromised in patients who have endometriosis-associated with pelvic pain compared to the other two groups [33]. The anxiety and depression in endometriosis patients was not correlated with the stage of the lesion nor the age of the patients [34]. In addition, it is hypothesized that the mental disturbance in endometriosis patients may increase the intensity of the pelvic pain associated with endometriosis, which in turn increases the psychological stress [35]. Taken together these findings, it might be plausible to treat endometriosis with no or little hormonal interventions. These options, such as OCP, have already been proved to cause depression that may exacerbate the psychological discomforts which endometriosis patients endure.

6.2 The use of anti-MMP therapies for endometriosis treatment

As mentioned above, introducing a hypo-estrogenic state appears to be universal in treating endometriosis, even with the undesirable side effects. Considering alternative approach through inhibiting MMPs may carry potential in reducing the complications associated with current medications used. Based on current data, several MMPs are highly expressed in ectopic lesion which positively correlated with lesion remodeling, angiogenesis, invasiveness, and establishment. The inhibition of MMPs by TIMPs has been used successfully in research setting which resulted in reducing the lesions survival by altering the ratio of MMPs to TIMPs [275]. Other than using the biological inhibitors of MMPs; a study showed that curcumin, a natural substance isolated from

curcuma longa, could increase the expression of TIMP-2 which in turns confines the activity of MMP-2 leading to less invasive lesions and hinders disease development [276].

Considering the hypothesis of endometriosis inflammatory origin, it is tempting to use anti-inflammatory agents to manage the burden of the disease. Treatment of mice with experimental endometriosis with lipoxin A4, an endogenous anti-inflammatory acid, showed decreased in the in the invasiveness and the size of the ectopic implants through the reduction of MMP-2 and MMP-9 expression [277]. Similarly, using inhibitor for NF- κ B (nuclear factor- κ B) was suggested method to treat endometriosis. NF- κ B is transcriptional factor that is expected to be over-activated in endometriotic cells, through TNF- α cytokine. Upon activation, NF- κ B controls the production of specific genes, including MMPs which enhance the angiogenesis and the invasion of the lesion tissues. Accordingly, pyrrolidine dithiocaramate (PDTC) was used to inhibit the action of NF- κ B, which results in downregulation of MMPs, reduction in the invasiveness of the ectopic lesion, and promoting apoptosis of endometriotic ectopic stromal cells [278]. Although limited in numbers, these studies suggest that MMP inhibitors may be beneficial but human studies clearly need to be conducted.

6.3 Blocking MMP action with anti-tumor necrosis factor- α therapies

TNF- α is a pro-inflammatory cytokine which has been considered as a significant player in the development of endometriosis. The peritoneal levels of TNF- α is elevated imposing a potential role in increasing the invasion of the retrograded endometrial cells [123, 244, 279, 280]. This is expected to occur due to its positive effect on MMPs levels leading to increased expression of MMPs in ectopic lesion. Therefore, blocking the action of TNF- α would be useful to target MMPs which could be a potential, non-hormonal- therapy to treat the disease. Anti- TNF- α has been used successfully in the management of some inflammatory diseases. In animal experimental models

of endometriosis, the use of Recombinant human TNF receptor: Fc fusion protein, (rh) TNFR: Fc, has been shown to limit the aggressiveness of the disease. Barrier and coworkers demonstrated that using etanercept, an anti-TNF- α antibody, subcutaneously in baboon model was effective in reducing the size and number of active red endometriotic lesions [281]. Similarly, a rat model of endometriosis was treated with etanercept and showed decreases in the size of implants compared to the control group [282]. More recently, the use of (rh) TNFR: Fc *in vitro* decreased the proliferation and adhesion of human endometrial stromal cells and decreased the levels of MMP-1. Similar results using rhTNFR:Fc were observed *in vivo*, using the mouse model of endometriosis where a reduction in the ectopic endometriotic lesion size was noted [283]. Unfortunately, the more recent use of rhTNFR:Fc has not been evaluated in humans, but the published data in animal models are promising and may support the use of anti- TNF- α as prospective therapy to treat and manage endometriosis.

7. Summary and Perspective

The pathophysiology of endometriosis is complicated and not well clear, which makes the process of finding a cure a hurdle task. Therefore, it may be rational if we consider endometriosis as a multi-factorial disease where a combination of factors, hormonal and immune factors, is involved to promote the attachment, aggressiveness and the angiogenesis of the ectopic lesion. The observation of the estrogenic stimulatory effects on the tissues drove the field to use the GnRHa as a major therapy to suppress the lesion growth and to control the disease; however, considering the patient's quality of life and the associated complications, this may not be the ideal treatment. On the other hand, the dysregulation of the immunity and inflammatory components is well established. MMP, which is regulated by cytokines, has been linked to enhance the survival and establishment of the lesion. Focusing on this pathway to treat endometriosis could be beneficial in

limiting the adhesion of the tissue even with continuous steroidal stimulation which may reduce the term of the disease and improves the patients' health.

Chapter V:

Concluding Statements and Future Directions

Endometriosis is defined as presence of glandular and stromal tissue in ectopic locations, primarily within the pelvic cavity. The process of lesion development initiates during menstruation which involves shedding of the endometrium that lines the uterus, followed by migration through the fallopian tubes, ectopic attachment/adhesion of retrogradely shed endometrial fragments, proliferation, and survival of the lesion. In this dissertation, we were interested in studying the modulators that induce the proliferation and survival of endometriotic lesion/endometriotic cells. Because of the important established role of miRNAs in endometriosis, we focused on detecting new targets of miR-451a to expand its pathway. The results of this study are expected to provide better understanding of the pathogenesis of the disease and identify potential therapeutic targets for endometriosis treatment.

1- Over-expression of miR-451a reduced the level of RPLP1.

In endometriosis, it is already established that miR-451a reduces cellular proliferation/survival by directly binding to and reducing the expression of genes including YWHAZ and MIF (Macrophage Inhibitory Factor) [131, 159]. Initially, we wanted to identify additional targets of miR-451a which may be relevant to the pathophysiology of endometriosis. As miR-451a is predominantly, if not exclusively, expressed by endometrial/endometriotic glandular epithelial cells [131] we utilized the endometriotic epithelial cell line, 12Z as our experiment model. We transfected 12Z cells with pre-miR-451a to integrate miR-451a protein targets using 2D gel analysis. From the dozens of proteins which were differentially expressed, we narrowed our focus to 25 proteins that were differentially expressed using the criteria previously described [284]. For this project, we selected RPLP1 (Ribosomal protein Large P1) as our protein of interest based upon its role as a potential mediator of cell proliferation/survival and the facts that the protein has not been described in the pathophysiology of endometriosis and its level of expression in the 12Z cell line. As the role of

RPLP1 in endometriosis is un-investigated, the available literature showed that RPLP1 is a constituent of ribosomal structure [170-173], further studies demonstrated elevation of RPLP1 in gynecological tumor [179], and it promotes cellular transformation and proliferation [178]. Thus, we hypothesized that RPLP1 plays a role in the proliferation of endometriotic lesions. Therefore, the focus of our study was to define the expression of RPLP1 in human endometriotic lesion and eutopic endometrial samples, study its function and regulation.

2- RPLP1 protein is elevated in endometriotic lesions:

The objective of this experiment was to examine the expression of RPLP1 in human endometriotic lesion and eutopic endometrium. RPLP1 transcript and protein was significantly higher in human endometriotic ectopic lesions compared to matched eutopic tissues from the same patient and compared to endometrium from women without endometriosis. Knowing that proliferation/survival are characteristics of endometriotic lesions, and considering the proliferative function of RPLP1, the elevation of RPLP1 in these specimens was logical and supports our working hypothesis. We also found positive association between RPLP1 and CCNE1 (Cyclin E1), a marker of proliferation, in the same lesion, which again supports our hypothesis. However, it should be emphasized that although net RPLP1 expression in lesion tissue was significantly elevated compared to eutopic endometrium, there was a great deal of heterogeneity among the levels of expression within individual lesions. It is well established that endometriotic lesion tissue are a heterogeneous tissue and this may be one reason that may hinder or delay the development of diagnostic/therapeutic targets. We believe the level of expression of RPLP1 by individual lesion tissue plays a physiologic/pathophysiologic role and is not due to different cell populations that make up individual lesions. We base this statement on the fact that we have evaluated the potential differences in the epithelial and stromal composition among samples by analyzing markers for

each cell type and found no significant differences [159] suggesting that differences in RPLP1 expression could not be attributed to an enrichment or reduction in the proportion of epithelial cell content compared with eutopic tissue.

This information leads us to believe that RPLP1 (and miR-451a) levels change as individual endometriotic lesions “age”. While it is impossible to evaluate this in humans, the use of animal models such as the baboon or rodent, would allow for such evaluation by inducing experimental endometriosis and then evaluate lesion content at different time points.

3- RPLP1 expression is essential to cell proliferation in cell model:

To confirm the role of RPLP1 in cell model, we knocked down the expression of RPLP1 in 12Z cells and found reduction in the level of CCNE1 (proliferating marker), and elevation of PTEN (Pro-apoptotic marker) of cells lacking RPLP1. Transient knockdown of RPLP1 showed reduction in the number of viable cells and cell survival. The results of this study support our initial hypothesis and agrees with previous studies which suggest that RPLP1 is important in cellular proliferation and cell survival.

In future, it would be interesting to see if RPLP1 inhibition causes also reduction in the number of ribosomes, which therefore triggering arrest in the global translation machinery and leads to cell death. If no significant change is detected in the number of ribosomes, it is interesting to examine the effect of RPLP1 knockdown in the translation of certain proteins necessary for cellular growth and survival. To confirm if RPLP1 has a central function in cellular proliferation, further studies could be developed to rescue RPLP1-depleted cells with RPLP1-ORF cDNA lentiviral particles, which theoretically would reverse the above morphologies and resume cellular growth and survival

4- miR-451a regulates the expression of c-Myc in 12Z cells:

At this point, we have already confirmed that forced expression of miR-451a causes a reduction of RPLP1 in 12Z cells. However, the process of miR-451a binding to the mRNA sequence of RPLP1 (3' UTR, 5' UTR, or other regions), which is essential for direct regulation of miRNAs, has not yet been confirmed. We looked at the bio-informatic resources available and found no evidence of such regulation. Therefore, we speculated that miR-451a controls the expression of RPLP1 indirectly, thus, we searched for up-stream regulators of RPLP1. While there are limited studies focusing on RPLP1 pathway, microarray-based study showed that RPLP1 is c-Myc responsive gene in rat fibroblast [200]. Interestingly, based on bio-informatic tools and previous studies, c-Myc, a universal transcription factor, is a direct target miR-451a [157, 158, 161]. Although this study was obtained from fibroblast cells (equivalent to the stromal cells in endometrium), the collective data was encouraging to suggest that miR-451a directly binds to c-Myc and this regulates the expression of RPLP1. Our findings showed that over-expression of miR-451a reduces Myc RNA and protein levels in 12Z cells. In addition, we demonstrated that Myc knockdown causes reduction in RPLP1 level, which validated that Myc is a potential regulator of RPLP1.

In the future, we want to confirm that miR-451a is a direct repressor of c-Myc and indirect regulator of RPLP1. This can be performed by luciferase reporter assay to determine if miRNA binds to the 3'UTR of target gene. In addition, since c-Myc is a transcription factor that binds to the promoter sequence of its target gene to control its expression, the use of chromatin immunoprecipitation (ChIP) assay is useful to examine if c-Myc binds to the RPLP1 promoter sequence. The results of these experiments, if positive, will confirm our findings. Furthermore,

it is important to note that RPLP1 may also be regulated by other intermediate proteins and/or other miRNAs, and not exclusively relying on Myc and miR-451a.

Moreover, it would be interesting to see if any of the other candidate proteins identified in the 2D-gel analysis are of Myc responsive genes.

5- Identification of potential therapeutic targets of endometriosis:

Because the main objective of expanding this pathway is to find new therapeutic targets for endometriosis; the need for further set of experiments *in vitro* and *in vivo* (using mouse, or baboon model for endometriosis) are required to find additional modulators in this pathway that are more specific to endometriosis. While RPLP1 is a novel finding in endometriosis pathophysiology, it may not be ideal to use RPLP1 inhibitor, if available, as method to reduce lesion development and treat the disease. Since RPLP1 is widely expressed in all cells and its expression is essential for ribosomal function and cellular survival, inhibiting its production/or function could be detrimental.

Similarly, there are several Myc inhibitors that are used in research settings to investigate the effect of myc suppression [1]. Yet, there are limitations in using these inhibitors in humans due to their toxic effects. Just recently, the FDA resumed the clinical trial on using Myc inhibitors such as APTO-253 to treat acute myeloid leukemia (AML) patients and high-risk myelodysplastic syndromes (MDS) [285]. In addition to the safety concerns regarding such inhibitors, the new medication should overcome the disadvantages of the current hormonal therapy of endometriosis such as depression, bone loss, and pregnancy prevention [56]. Therefore, further studies are needed to address the relation between RPLP1 pathway and

ovarian hormonal regulation. Overall, the use of RPLP1 or Myc inhibitors could be promising if specific targeting of endometriotic lesions is feasible.

Further studies to expand the pathway of miR-451a may aid in treatment development. For example, miR-451a is potential repressor of matrix metalloproteinases (MMP-9 and MMP-2) in cancer [286, 287]. If such regulation occurs in endometriosis is yet to be defined. However, the expression of MMPs in endometriosis is well-established and discussed in depth in chapter 4 of this dissertation. The function of MMPs and their inhibitors (TIMPs) is to degrade the extracellular matrix and modify tissue structure in a controlled fashion [229]. The use of MMP biological inhibitors (TIMPS) was investigated in animal model of endometriosis and resulted in significant reduction of the size of endometriotic lesions [275], suggesting that such an approach may be feasible.

In conclusion, our findings indicate that proliferation of endometriotic lesions is potentially promoted by a RPLP1-dependant pathway. In these lesions, the low level of miR-451a induces the expression of its target gene, c-Myc, and that in turn up-regulates the expression of RPLP1, which increases cellular survival.

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